University of Alberta

Feeding a protective semi-purified diet to diabetes prone rats improves T lymphocyte phenotype profiles

by

Susan Glen

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Masters of Science

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall 1996

National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file *Votre référence*

Our file *Notre référence*

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-18264-9

Canada

University of Alberta

Library Release Form

Name of Author: Susan Glen

Title of Thesis: Feeding a protective semi-purified diet to diabetes prone rats improves T lymphocyte phenotype profiles

Degree: Masters of Science

Year this Degree Granted: 1996

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly, or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

Susan Glen

108-111th Street
Saskatoon, SK
S7N 1S7

Date submitted: Oct 4/96

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The effect of diet on lymphocyte phenotypes in young, diabetes prone and normal rats submitted by Susan Glen in partial fulfilment of the requirements for the degree of Masters of Science in Nutrition and Metabolism.

Catherine J Field _____ Dr. Catherine J. Field (Supervisor)

_____ Dr. M. Thomas. Clandinin

Michael I. McBurney _____ Dr. Michael I. McBurney

Edmond A Ryan _____ Dr. Edmond Ryan

Date: October 2, 1996

Dedication

This thesis is dedicated to my mother and father for their constant encouragement and support of everything I have ever done, and to the memory of Graham Davies.

Abstract

A study was undertaken to establish the effect of diets (NIH, casein, soy) producing different diabetes incidence in diabetes-prone (BBdp) rats, on immune cells. Lymphocyte phenotypes were determined at 14 and 30 days of age. Feeding a protective casein based diet increased the proportion of T cells (OX19+, $CD8^+$ and $CD4^+$) at day 30 to more closely resemble that of non-diabetes prone rats, by T cells. The CD4/CD8 ratio in the BBdp rats fed the casein and soy diets did not differ from normal rats at day 30 . Differences in T and B cell proportions were also seen at day 14 in pups whose dams were fed different diets. Growth rate was different between the three groups with animals fed the NIH diet weighing more. A combination of early growth and an increased proportion of T cells may reduce the incidence of diabetes in casein fed rats.

Acknowledgments

Firstly, I would like to thank my supervisor Dr. Catherine Field for sharing her knowledge and insight in all areas of my research. As well for her constant support and encouragement in my studies as well as personally.

I would also like to thank Dr. Tom Clandinin and Dr. Mike McBurney for being on my supervisory and examining committee. In addition Dr. E. Ryan for serving as my external examiner.

I would also like to acknowledge Jody for her help with animal care and Donna Claerhout for technical assistance. Also Susan Goruk and Dr. Goh for their knowledge about anything and everything in the lab

Lastly I extend heartfelt thanks to my fellow students for friendship, support and lots of laughter. In particular Raylene Reimer, Stephanie Cook and Stephanie Conrad who all helped me through the last three years.

Table of Contents

Chapter One - Literature Review		Page
I.	**Introduction**	1
	i. Human Insulin Dependent Diabetes Mellitus	1
	ii. The Bio-Breeding Rat as a model of IDDM	1
II.	**Genetics**	1
	i. Human IDDM	1
	ii. The BB Rat	2
III.	**Immunology**	3
	i. Human IDDM	3
	ii. The BB Rat	6
IV.	**Environmental Factors in the Etiology of IDDM**	9
	i. Human IDDM	9
	i.a) Early infant diet as an environmental factor in the etiology of IDDM	10
	i.b) The effect of early diet on growth	12
	ii. The BB Rat	12
	ii.a) Diet as an environmental factor in the etiology of IDDM in the BB rat	12
	ii.b) The effect of diet on growth	15
Chapter Two - Research Plan		16
	Rationale	16
	Hypotheses	17
	Research Objectives	17
Chapter Three - Materials and Methods		18
	i. Chemicals	18
	ii. Animals and Diets	18
	iii. Lymphocyte Isolation	20
	a) Blood	20
	b) Spleen, Thymus, Mesenteric Lymph Nodes	20
	iv. Mononuclear Cell Phenotyping	21
	v. Statistical analysis	22
Chapter Four - BBdp Results		23
	Animal Characteristics	23
	Lymphocte Phenotypes	26
	Effect of Age and Diet on Splenocyte Phenotypes	44

Chapter Five - BBn Results 50
Animal Characteristics] 50
Lymphocte Phenotypes 53
Effect of Age and Diet on Splenocyte Phenotypes 70

Chapter Six - Discussion 76

Chapter Seven - Bibliography 89

Tables

3.1	Diet compositions	19
3.2	Monoclonal antibody specificity	21
4.1	Day 14 Animal Characteristics - BBdp	24
4.2	Day 30 Animal Characteristics - BBdp	25

Effect of Age and Diet on Splenocyte Phenotypes (BBdp)

4.3.1	W3/25 (CD4)	46
4.3.2	OX8 (CD8)W3/13	46
4.3.3	CD4/CD8 ratio	46
4.3.4	OX19	47
4.3.5	W3/13	47
4.3.6	OX19/W313 ratio	47
4.3.7	OX12	48
4.3.8	OX33	48
4.3.9	3·2·3	48
4.3.10	OX42	49
5.1	Day 14 Animal Characteristics - BBn	51
5.2	Day 30 Animal Characteristics - BBn	52

Effect of Age and Diet on Splenocyte Phenotypes (BBn)

5.3.1	W3/25 (CD4)	72
5.3.2	OX8 (CD8)W3/13	72
5.3.3	CD4/CD8 ratio	72
5.3.4	OX19	73
5.3.5	W3/13	73
5.3.6	OX19/W313 ratio	73
5.3.7	OX12	74
5.3.8	OX33	74
5.3.9	3·2·3	74
5.3.10	OX42	75

Figures

BBdp Day 14 Splenocyte Phenotypes

Page

4.1.1 OX19 29
4.1.2 W3/13 29
4.1.3 OX19/W313 ratio 29
4.1.4 W3/25 (CD4) 30
4.1.5 OX8 (CD8) 30
4.1.6 CD4/CD8 ratio 30
4.1.7 OX12 31
4.1.8 OX33 31
4.1.9 3·2·3 31
4.1.10 OX42 32

BBdp Day 30 Splenocyte Phenotypes

4.2.1 OX19 33
4.2.2 W3/13 33
4.2.3 OX19/W313 ratio 33
4.2.4 W3/25 (CD4) 34
4.2.5 OX8 (CD8) 34
4.2.6 CD4/CD8 ratio 34
4.2.7 OX12 35
4.2.8 OX33 35
4.2.9 3·2·3 35
4.2.10 OX42 36

BBdp Day 30 Mesenteric Lymphocyte Phenotypes

4.3.1 OX19 37
4.3.2 W3/13 37
4.3.3 OX19/W313 ratio 37
4.3.4 W3/25 (CD4) 38
4.3.5 OX8 (CD8) 38
4.3.6 CD4/CD8 ratio 38
4.3.7 OX12 39
4.3.8 OX33 39

BBdp Day 30 Blood Lymphocyte Phenotypes

4.4.1 OX19 40
4.4.2 W3/13 40
4.4.3 OX19/W313 ratio 40
4.4.4 W3/25 (CD4) 41

Page

4.4.5 OX8 (CD8) 41
4.4.6 CD4/CD8 ratio 41
4.4.7 OX12 42
4.4.8 OX33 42
4.4.9 3·2·3 42
4.4.10 OX42 43

BBn Day 14 Splenocyte Phenotypes

5.1.1 OX19 55
5.1.2 W3/13 55
5.1.3 OX19/W313 ratio 55
5.1.4 W3/25 (CD4) 56
5.1.5 OX8 (CD8) 56
5.1.6 CD4/CD8 ratio 56
5.1.7 OX12 57
5.1.8 OX33 57
5.1.9 3·2·3 57
5.1.10 OX42 58

BBn Day 30 Splenocyte Phenotypes

5.2.1 OX19 59
5.2.2 W3/13 59
5.2.3 OX19/W313 ratio 59
5.2.4 W3/25 (CD4) 60
5.2.5 OX8 (CD8) 60
5.2.6 CD4/CD8 ratio 60
5.2.7 OX12 61
5.2.8 OX33 61
5.2.9 3·2·3 61
5.2.10 OX42 62

BBn Day 30 Mesenteric Lymphocyte Phenotypes

5.3.1 OX19 63
5.3.2 W3/13 63
5.3.3 OX19/W313 ratio 63
5.3.4 W3/25 (CD4) 64
5.3.5 OX8 (CD8) 64
5.3.6 CD4/CD8 ratio 64
5.3.7 OX12 65
4.3.8 OX33 65

Page

5.4.1 OX19 66
5.4.2 W3/13 66
5.4.3 OX19/W313 ratio 66
5.4.4 W3/25 (CD4) 67
5.4.5 OX8 (CD8) 67
5.4.6 CD4/CD8 ratio 67
5.4.7 OX12 68
5.4.8 OX33 68
5.4.9 3·2·3 68
5.4.10 OX42 69

6.1 Model 86

List of Abbreviations

IDDM	Insulin Dependent Diabetes Mellitus
BBdp	Bio-breeding rat (diabetes prone)
BBn	Bio-breeding rat (non-diabetes prone)
SP	Semi-purified
NP	Non-purified
CAS	casein-based semi-purified diet
SOY	soy-based semi-purified diet
NIH	NIH-07 chow
CMP	Cow's milk protein
NK	Natural killer cells
MHC	Major Histocompatability Complex
HLA	Human Leukocyte Antigen
Ig	Immunoglobulin
TNF	Tumor Necrosis Factor
IL	Interluekin
IFN	Interferon

Chapter One - Literature Review

I. Introduction

i. Human Insulin dependent diabetes mellitus

Insulin dependent diabetes mellitus (IDDM) is an autoimmune disease that affects millions of people world-wide. IDDM involves the permanent destruction of the insulin secreting β-cells of the pancreas. Treatment for IDDM involves the provision of exogenous insulin for life and even with this treatment complications often occur, including blindness, kidney damage and atherosclerosis. There is a genetic predisposition to the disease, however not all susceptible individuals develop IDDM (Wilkin T and Armitage M, 1986). In monozygotic twins the concordance rate is less than 50% (Peakman et al., 1993; Barnett et al., 1981). These observations imply that there are environmental factors involved in the etiology of IDDM.

ii. The Bio-Breeding Rat as a model of IDDM

The bio-breeding (BB) rat is an animal that spontaneously develops a syndrome similar to human IDDM (Parfrey et al., 1989; Yale and Marliss, 1984). As in human IDDM, the BB rat syndrome appears to be autoimmune in nature and results in the permanent destruction of the insulin secreting β-cells of the pancreas (Dean et al., 1987). As in IDDM there is a large genetic component in the disease etiology (Fuks et al., 1988), but, like the human case, not all BB diabetes prone (BBdp) rats develop the disease (Colle et al., 1992). This indicates that some enviornmental factor acts on a background of genetic susceptibility to produce the IDDM-like syndrome in the BB rat. The BBdp rat strain developed spontaneously from the BB rat strain now known as the BBn (non-diabetic) strain. BBn animals are used for control animals in studies with BBdp rats.

II. Genetics

i. Human IDDM

There is without question a genetic component involved in the etiology of IDDM. As stated above, there is a concordance rate of 40%-50% in monozygotic, identical twins as compared to a 6% concordance rate between non-monozygotic siblings (Thomson et al., 1988).

The HLA (Human Leukocyte Antigen) region of the genome found on the short arm of chromosome six seems to be the region most closely connected to an individual's susceptibility to develop IDDM. More specifically the HLA region that encodes for class I and class II molecules, tumor necrosis factor (TNF) and complement has been shown to correlate with the presence or absence of IDDM (Dorman et al., 1991). The class I and class II molecules are those responsible for the presentation of antigens to T-cells. Caucasian individuals who possess the genes for the DR3 and/or DR4 form of the class II molecules are at a much higher risk for IDDM. Ninety percent of Caucasians with IDDM carry the DR3/DR4 as compared to only 45% of non-diabetic individuals (Thorsby et al., 1991). In addition those who possess genes for the DR2 form of the molecule are rarely found to have the disease (Svejgaard et al., 1983). This association may be linked to a companion molecule which is associated with the DR class II molecules and is encoded in the same region of chromosome six. This molecule is the DQ molecule. It has been shown that the DQw8 form which associates with DR4 is correlated to increased susceptibility (Owerbach et al., 1983) while the DQw6 form, associated with the DR2 molecule, is correlated to protection from IDDM (Tiwari and Terasaki, 1985). This correlation may be due to a change in one residue of the molecule located at position 57. The gene for DQw6 codes for aspartic acid at this position whereas the gene for Dqw8 codes for a non-aspartic acid molecule (Todd et al., 1987). Residue 57 is located in the antigen binding cleft of the class II molecule and the importance of this will be discussed in the section III.

The genes and their products mentioned above are those with the greatest correlation to IDDM susceptibility in Caucasians. The fact that the HLA complex is the area where they are located gives support for IDDM as an auto-immune disease. Other genes, also located in the HLA complex are correlated with a higher risk for individuals of other races.

ii. The BB Rat

In the BB rat the majority of the genes connected with susceptibility to or protection from the diabetic syndrome are found in the major histocompatibility complex (MHC) region of the genome (Colle et al., 1981). This region is analagous to the HLA region in humans and is responsible for the production of class I and

class II molecules which are necessary for the recognition of an antigen by a T-cell (Abbas et al., 1991).

The particular allele in BBdp rats that is most closely associated with susceptibility or protection is the RT1 allele. Susceptibility to the disease requires animals to be homozygous for the $RT1^u$ haplotype or heterozygous with $RT1^u$ in combination with $RT1^a$, $RT1^l$ or $RT1^b$ (Fuks et al., 1991; Colle et al., 1981).

The RT6 gene is also associated with the diabetic syndrome in these rats (Rossini et al., 1986; Burstein et al., 1989). This gene codes for a maturation marker found on the majority of T cells in the rat. There are two alleles of this gene RT6.1 and RT6.2. It was believed that BBdp rats lacked this marker on their T-cells (Greiner et al., 1986). Although the gene has been found to be normal (Thiele et al., 1989), and is transcribed and translated in these rats, its level of expression on T-cells is ≤ 10% of BBn T-cells (Crisa et al., 1993). This defect in expression appears to be thymus independent (Thiele et al., 1989) and is proposed to be the result of a decreased lifespan of T-cells in the BBdp animal, since RT6 is a maturational marker (Sarkar et al., 1992). Diabetes development is at least partially dependent on this lack of expression and will be discussed further in section III ii.

III. Immunology

i. Human IDDM

Although it is widely accepted that IDDM is an autoimmune disease (Nerup and Lernmark A,1981; Castano and Eisenbarth, 1990) the exact cause and nature of the immuno-pathogenesis has yet to be determined. There is evidence that both the humoral, and cell-mediated arms of the immune system are required for, and involved in, the autoimmune process.

Many different antibodies have been associated with IDDM. In newly diagnosed diabetic individuals, levels of islet cell antibodies (ICA) as well as insulin antibodies (IA) are found to be elevated when compared to non-diabetic individuals (Casali et al., 1990; Dahlquist et al., 1992). More importantly, the frequency of B cells able to produce IA when stimulated is significantly higher in diabetic individuals. The IA antibodies produced are a high affinity monoreactive IgG isotype compared to low affinity polyreactive antibodies of IgA, IgM and IgG isotypes found in non-diabetic individuals (Casali et al., 1990). The response in diabetic subjects

(monoreactive, high affinity IgG) is more indicative of a specific immune response with memory characteristics (Abbas, 1991) than that of non-diabetic subjects (polyreactive, low affinity IgM, IgA, IgG) which is believed to represent a natural population of somewhat protective autoantibodies (Casali and Notkins, 1989; Ternick and Avrameas, 1986).

Both ICA and IAA could be involved in the autoimmune destruction of the pancreas by binding to proteins of the islet cells, IAA to insulin, and ICA to a 64 kD protein found on the β-cell of the pancreas (Baekkeskov et al., 1990). Once bound, the antibodies could initiate an antibody response resulting in the destruction of the antibody-bound cells via complement or by recruitment of cytotoxic cells and macrophages.

The 64kD islet cell protein is believed to be glutamate decarboxylase (GAD) (Atkinson & Macleran, 1993) and it has yet to be determined conclusively whether or not ICA are directly involved in disease process. However the level of these antibodies is currently used in prevention studies to predict diabetes risk in first degree relatives of diabetic individuals (Eisenbarth et al., 1993).

The major regulator of self-tolerance and therefore autoimmunity appears to be the cellular arm of the immune system (Abbas et al., 1991). Although humoral immunity may be important once the disease process is initiated (as discussed above), it is likely that the triggering event in the autoimmune process of IDDM is mediated through a defect or defects in an individual's cellular mechanism of self-tolerance. Self-tolerance is maintained by deletion of, or induction of anergy in autoreactive T-cells during their development (Abbas et al., 1991).

There are many components of the cell-mediated immune response that may be involved in the immuno-pathogenesis of IDDM. The T-cells of these individuals may be more easily and non-specifically activated than those of non-diabetic individuals (Tun et al., 1994; Alviggi et al., 1984; Miyazaki et al., 1995). This alone could lead to an inappropriate immune response since these cells could activate autoreactive T or B cells that would not normally receive these signals (Abbas et al., 1991). This phenomenon could result from abnormal activation by some factor, such as a viral infection (Gamble, 1980), or by a lack of suppressor activity by the immune system (Abbas et al., 1991). Although individuals with IDDM may also have an increased risk of developing other autoimmune diseases (Wertman et al., 1992;

Gadd et al., 1992), which supports the theory of generally increased activation or decreased suppression, IDDM with its very specific autoimmunity usually occurs alone. Therefore, other mechanisms must play a role in the disease process.

Recently, various cytokines produced by monoytes and T-cells have been implicated in IDDM (Lorini et al., 1995; Cavallo et al., 1992; Ohno et al., 1993). Tumor necrosis factor alpha (TNF-α), a cytokine produced by macrophages, natural killer (NK) cells and some activated T cells (Abbas et al., 1991), has been implicated in IDDM (Lorini et al., 1995; Ohno et al., 1993). Initially it was believed to be a causative factor in IDDM as TNF-α alone, or in combination with other cytokines can directly impair β-cell function (Lorini et al., 1995). However these findings have not been consistent. One study (Lorini et al., 1995) reported lower plasma levels in diabetic patients than in healthy individuals, while another (Cavallo et al., 1991) found higher cytokine concentrations in newly diagnosed patients compared to non-diabetic subjects. However, there was no corresponding increase in bio-activity of the cytokine when tested for using a cytotoxicity assay.

Elzirik et al., (1994) studied the ability of various cytokines to induce nitric oxide (NO) mediated islet damage. Individual cytokines were unable to induce NO production however IL1-β in combination with IFN-γ and TNF-α did increase the production of NO. Interestingly, this increase in NO did not impair the function of the islets to release insulin *in vitro*. Exposure to the cytokine combination over six days led to a decrease in glucose-induced insulin release but no apparent destruction of the β-cells. Therefore these cytokines appear to suppress the function of the islets rather than destroy them. This suppression was shown to be independent of NO production. It is possible that this impaired islet function may lead to eventual islet cell damage as human islet cells can be damaged by increased glucose concentrations (Elzirik et al., 1992).

Clearly cytokines play a major role in any physiological process that is mediated by the immune system. To date, the data about these biochemical mediators is inconclusive. This is in large part due to the fact that there is considerable *in vitro* data on individual cytokines but very little *in vivo* data. The ever-increasing knowledge regarding cytokines will hopefully lead to a complete understanding of their relationship to diabetes.

ii. The BB Rat

The BB rat shows many immune irregularities that may or may not be related to and/or necessary for disease development and progression. As in the human disease there is a significant increase in the number of auto-antibodies in the BBdp rats as opposed to the control rats (Dyrberg et al., 1984). However it has been established that it is abnormalities in the cellular arm of the immune system which lead to the development of IDDM in these rats, since animals thymectomized prior to 30 days do not develop diabetes (Like et al., 1982; Like et al., 1986).

The most dramatic of these abnormalities is an extreme lymphopenia which is characterized by a decrease in total lymphocytes with the largest deficiency in the number of T-cells (Yale and Marliss, 1984; Jackson et al., 1983). This deficiency occurs in all measurable subsets of T-cells. However the CD8+ cytotoxic/suppressor cells are most affected, as virtually no cells of this subset are found in peripheral circulation. Lymphopenia is present from birth and becomes more pronounced with age due to a reduced expansion of T-lymphocytes compared to BBn rats (Yale et al., 1985). In addition to their overall lymphopenia, BBdp rats have virtually no circulating T-cells that are $RT6^+$; RT6.1 being a marker found on almost all T-cells in the BBn rat (RT6.2 is found in other rat strains).

Many studies have examined which cell types, alone or in combination, may be necessary for diabetes to develop in BBdp rats. There are two main methods used to examine this. One is to deplete young BBdp rats of specific subsets of cells by injecting monoclonal antibodies that are cytotoxic to the desired cell subset, and then monitor for diabetes occurrence. The other is to inject young BBdp rats with specific subsets of mitogen stimulated cells, isolated from older, acutely diabetic BBdp rats and monitor for diabetes to occur at an earlier age than normal; this type of experiment is termed "adoptive transfer".

Using these methods $CD8^+$ T-cells have been shown to be required for diabetes development (Edourd et al., 1993; Like et al., 1986; Ellerman et al., 1993). In addition, Barlow and Like (1992) showed $CD2^+$ cells, particularly the $CD4^+$ T-cell subset of this group to be necessary for diabetes. This is confirmed by the observation that extremely low numbers of activated $CD4^+$ cells can induce adoptive transfer of diabetes from diabetic donors to young BBdp rats (Metroz-Dayer et al., 1990)

Natural killler cells were initially thought to play an important role in the pathogenesis due to the fact that their relative percentage compared to BBn rats is higher and activity is increased (Woda & Biron, 1986). As well, they have been shown to be cytotoxic to islet cells *in vitro* (Nakamura et al., 1990; MacKay et al., 1986). However deletion of NK cells from BBdp rats does not change the incidence of diabetes in these animals (Ellerman et al., 1993; Edourd et al., 1993). Therefore NK cells may participate in the disease process but do not appear to be necessary for it to occur.

Macrophages have been theorized to be potentially damaging to β-cells, as NO produced by these cells has been shown to potentiate cytokine-induced β-cell destruction *in vitro* (Suarez-Pinchon et al., 1994). As well, the administration of silica to 60 day old BBdp rats decreases the incidence of diabetes in these animals (Oschilewski et al., 1985). Silica has a specific action against macrophages by destroying them or rendering them non-functional (Lowrie, 1982). Therefore the results of the silica study appeared to show that macrophages play a significant role in the disease pathogenesis. However it is important to note that other changes took place in the silica treated rats. They were found to have enlarged parapancreatic lymph nodes and granuloma tissue in the connective tissue of the gut and pancreas, and larger spleens but fewer cells per gram of spleen (Oschilewski et al., 1985). Also, although body weights of the two groups were the same prior to silica treatment, growth following the treatment was diminished in the silica group leading to significantly lower body weights in these animals at both 120 and 160 days of age (Oschilewski et al., 1985).

In contrast to the data supporting the role of macrophages in diabetes it as also suggested they may be involved in suppressing the autoimmune process occurring in BBdp rats, as their removal from adoptive transfer injections leads to a higher diabetes incidence (Metroz-Dayer et al., 1990).

Without question T-cells that express RT6.1 play an important role in protecting animals from diabetes development. The BBn (resistant) strain which has normal numbers of $RT6^+$ T-cells (compared to other rat strains) can be induced to develop insulitis and/or diabetes by depleting this cell population (starting at 30 days of age) using anti-RT6 antibody injections (Greiner et al., 1987; Jaing et al., 1990). Another study concluded that RT6 depleted BBn rats were susceptible to diabetes

development but required an additional immunological stimulus to initiate the disease (Like, 1990). This latter study was conducted in a SPF barrier colony, so it is possible that the normal housing conditions in the former study provided the additional stimulus through common antigens. Two of the studies, (Greiner et al., 1987; Like, 1990), found that adoptive transfer of diabetes using injections of activated spleen cells from RT6 depleted BBn rats into 30 d BBdp rats, was successful.

Research using this animal model has shown that there is a critical period in early life in which an animals susceptibility to diabetes can be altered. An example of this is starting BBn RT6 depletion at different ages. Depletion started at 30 days induces diabetes but there was no effect when depletion was started at 60 days, even though depletion rates were the same (Greiner et al., 1987). These differences also held for the adoptive transfer experiments; stimulated RT6 depleted cells from 60 d animals did not accelerate the disease process when injected into 30 d BBdp animals (Greiner et al., 1987).

As in the human disease various cytokines may be involved in the disease process. TNF-α production by activated macrophages is upregulated prior to insulitis occuring in these rats (Rothe et al., 1990). However it is difficult to determine the effect of this excess TNF-α as rats injected with TNF-α from week 4 to week 27 were protected from the disease (Satoh et al., 1990). Therefore TNF-α appears to have the potential for both a protective and/or causative role in BBdp rats. IFN-α, a cytokine that is produced by islet cells, is also found to be upregulated prior to diabetes onset in BBdp but diminishes after the onset of the disease, likely due to the destruction of the cells which produce it (Haung et al., 1994). This experiment also demonstrated that inducing increased levels of IFN-α by injections of poly I/C (polyinosinic-polycytidilic acid) accelerated the development of diabetes in BBdp rats and led to the disease occuring in BBn rats. This induction of IFN-α must occur prior to 55 days of age to change the course of disease in these animals. BBdp rats treated with anti-IFN sera show a slight delay in the onset of the disease, however this treatment does not prevent the disease from occurring (Ewel et al., 1992). IL1-β accelerated the onset of the disease when given via intra-peritoneal injections. This could be a direct effect as this cytokine has been shown to produce specific β-cell

lysis when used to perfuse an intact pancreas (Wogensen et al., 1990). There is also potential for cytokines to be involved in protecting the β-cells from damage as IL1-β and TNF-α induce the release of free radical scavengers in islets (Sandler et al., 1991; Borg et al., 1992; Dayer-Metroz et al., 1992).

The study of cytokines and their effects is difficult and often produces contradictory results, as seen above. Results often differ between *in vivo* and *in vitro* studies as cytokines rarely act alone but rather in concert with each other. As stated in relation to the human disease it is highly probable that they do play a role in IDDM and further study would eventually elucidate this role.

IV. Environmental Factors in the Etiology of IDDM

i. Human IDDM

The same evidence that shows genetics play a role in the development of IDDM (as discussed in section *ii.*), also indicates that environmental factors must be involved. If this were not the case, the concordance rate in monozygotic twins would be much higher than 50% (Peakman et al., 1993).

Statistics from population studies give strong support for the environmental role in the disease process. The risk of developing IDDM is extremely variable between geographical area locations. For example Finland has an incidence rate of 28.6 cases per 100,000, whereas Japan's rate is 0.8 per 100,000 (LaPorte, 1985). Canada's rate falls between these two, at 9/100,000 (LaPorte, 1985). As well there is a difference in the incidence of IDDM between peoples residing in their traditional geographical area and those of the same genetic pool residing in a non-traditional area. An example of this is the different prevalence rates of Japanese children living in Japan or Hawaii. In the former, the rate is 11.8/100,000 and in the latter it is 43/100,000 (Mimura, 1982). There have also been drastic increases in IDDM incidence in genetically stable populations over periods of time, too short to allow major genetic changes. In Finland the incidence rate rose from 13/100,000 to 33/100,000 between the early 1950's and 1980's (Reunanen and Akerblom, 1985).

The phenomena described above have been attributed to various environmental factors. One of these is viral infection. The viruses Cocksackie B4 and rubella have both been linked to diabetes by the fact that many new cases of

IDDM are reported following an infection of one of these viruses (Yoon, 1979; Muser, 1978; Gamble DR, 1980).

The strongest link between environment and IDDM has been shown in studies relating early diet and feeding practices to the development of the disease later in life.

i.a. Early Infant Diet as an Environmental Factor in the Etiology of IDDM

Many retrospective studies, with a variety of social and ethnic groups have shown a negative correlation between the duration of breast feeding as an infant, and IDDM occurrence in adolescence (Mayer et al., 1988; Kostraba et al., 1992; Borch-Johnsen et al., 1984; Blom et al, 1989). In a 1994 meta-analysis of many of these studies, Gerstein concluded that children who were breast fed for less than three months had an odds ratio (OR) for IDDM of 1.43 (1.15-1.77) compared to those breast fed for greater than three months. Therefore, it is reasonable to predict that although the disease usually manifests itself in early adolescence, events in infancy could affect the disease process which is thought to be initiated many years prior to the onset of overt IDDM (Gorsuch et al., 1981).

The duration of breast feeding could affect the pathogenesis of IDDM in an individual for a variety of reasons. Breast milk contains many factors which contribute to the development of the immune system in an infant. These include immunoglobulins, cytokines, complement factors, lymphocytes and macrophages (Ogra and Ogra, 1978). These immune components may be important in the normal development of an infant's immune system since they confer passive immunity to the infant while their own system is maturing. This will be discussed further in section B, however it may be that this passive immunity via breast milk offers some protection against the development of an inappropriate autoimmune response in genetically susceptible individuals.

Breast feeding, if it is the exclusive source of nutrition for an infant, may also confer protection by decreasing the potential of exposure to foreign antigens via solid food or formula. Many of the retrospective studies that have examined the relationship between breast feeding and diabetes incidence have also looked at the correlation between the incidence of IDDM and the exposure to solid food and/or formula, specifically cow's milk. One study has shown that individuals with IDDM

were exposed to solid foods earlier than non-diabetic individuals (Kostraba et al., 1993). This same study and another (Kostraba et al., 1992) have shown this relationship also holds for specific ethnic groups. Even more research points to a correlation between an earlier age of introduction to formula (mainly cow's milk based) and increased incidence of diabetes (Kostraba et al., 1992; Kostraba et al., 1993; Virtanen et al., 1994). As well there have been correlations made between consumption of cow's milk and incidence rates in specific geographical areas (Fava et al., 1994; Dahl-Jorgensen et al., 1991). Using meta-analysis, Gerstein (1994), reported an odds ratio of 1.63 (1.22-2.17) for IDDM in children who were fed cow's milk before 3-4 months compared to those not exposed to cow's milk in this period. This correlation was shown to be stronger in children who are considered "high risk" due to their genetic profile (Kostraba et al., 1993).

Several theories have been developed to explain this correlation. One is that there is a particular fraction of cow's milk that may be responsible. Bovine serum albumin (BSA), and some of its specific peptide sequences, are the components most commonly thought to be diabetogenic (Karjalainen J et al., 1992; Virtanen SM et al., 1994; Cheung R et al., 1994; Robinson BH et al., 1993). One peptide sequence in BSA, the ABBOS peptide is thought to be simiiar to a peptide expressed on the β-cell, p69. This theory holds that antibodies made to the ABBOS peptide are able to cross-react with p69 and lead to the destruction of the β-cell (Karjalainen et al., 1992). There is some evidence for this theory, as in some studies newly diagnosed IDDM patients have an elevated level of anti-ABBOS antibodies (Karjalainen et al., 1992). As well, ABBOS peptides found in an individual with IDDM have not always been able to stimulate an immune response by that individual's lymphocytes (Atkinson et al., 1993).

There have also been studies which show a significant correlation between antibodies to lactoglobulin and diabetes (Virtanen et al., 1994; Dahlquist et al., 1992). These correlations may be meaningful, or they may simply be the result of a stimulated immune system in diabetic patients. Other possibilities include a relationship with early introduction of cow's milk via formula, or current consumption of cow's milk (at time of anti-body testing), as both of these variables have been correlated to significantly higher levels of antibodies to cow's milk proteins in diabetic and non-diabetic individuals (Kletter et al., 1971; Taino et al., 1988; Virtanen et al.,

1994). From these findings it is apparent that more extensive research is needed to determine if dietary antigens are involved in the pathogenic process.

i.b. The Effect of Early Diet on Growth

A significant difference between infants who are breast fed and those who are bottle fed is their rate of growth in the first year of life. Formula fed infants grow more rapidly than those who are breast fed and this difference remains after the introduction of solid foods (Heinig et al., 1993; Dewey et al., 1992; Roche et al., 1993; Pathak et al., 1993). This difference in growth rate is likely due to formula fed infants consuming more energy than those that are breast fed) and more protien (Heinig et al., 1993), in the first year of life. This may be because in breast fed infants solid food replaces the milk source and it is usually additional in formula fed infants (Heinig et al., 1993). There is evidence to suggest that the differences in body weight between these two groups is due to increased fat gain in formula fed infants over breast fed (Dewey et al., 1993).

ii. The BB Rat

As in the case of human IDDM, genetic susceptibility is necessary for the disease to develop, yet not all BBdp rats become diabetic (Colle et al., 1992). Due to the fact that rat colonies have much more controlled environments than free living humans, variations in environmental influences can be studied more easily.

IDDM in the BB rat has also been linked to viral infections. In contrast to humans, there is evidence for both a protective and causative role for viruses (Scott and Marliss, 1991). However one of the main environmental factors that has been linked to the disease etiology is diet and early feeding practices.

ii.a. Diet as an enviornmental factor in the etiology of IDDM in the BB rat

The BB rat has proven to be an exceptional model to study the effects of diet on diabetes incidence. Dietary manipulation can greatly affect the incidence of the disease in these animals (Elliot and Martin, 1984; Scott et al., 1985; Daneman et al., 1987; Issa-Cergui et al., 1989; Hoorfer et al., 1992).

The first studies to show a diet effect compared incidence rates of rats weaned on to a commercial, cereal based, non-purified diet (chow) to those fed a

semi-purified (SP) diet (Scott et al., 1985; Issa-Cergui et al., 1989). The results showed a significant drop of at least 50% in the rats fed the semi-purified diet (Scott et al., 1985; Issa-Cergui et al., 1989; Scott and Marliss, 1991; Hoorfer et al., 1992). Both the timing and duration of exposure to the SP diet were found to be critical for the effect to occur. The diet must be introduced before 30 days of age and continue for at least 100 days in order to decrease the diabetes incidence (Issa-Cergui et al., 1989; Scott and Marliss, 1991). These results support the likelihood of a critical period in early life being important in the development of the disease. This corresponds to the human studies discussed above, which also indicate that early diet practices have an effect on the occurrence of IDDM later in life.

In response to the results of these studies, many investigators have attempted to determine which component of the chow diet might be diabetogenic. Studies examining the protein source of the rat diet have shown variable results. Several have looked at the role that cow's milk proteins (CMP) may play in the disease pathogenesis. One study (Elliot and Martin, 1984) showed that weaning pups onto a diet utilizing synthetic amino acids as the protein source, gave an incidence rate of 15%. Pups weaned to an identical diet with 1% skim milk powder added, had an incidence rate of 50%, which was the level normally seen in the chow fed BBdp rats in this colony. Another study (Daneman et al., 1987) following a similar protocol, weaned animals to one of three different diets: chow without CMP, chow with 1% CMP or regular chow which contains 0.3-0.4 % CMP. They found that the animals weaned to chow without CMP had a significantly lower incidence of diabetes than those animals weaned to regular chow or chow with 1% CMP added.

Milk protein contains as many as 25 different proteins, with the main sources being casein, lactoglobulin and lactalbumin (Scott et al., 1994). Different investigators have attempted to elucidate which of these fractions, when present in the diet may lead to an increased risk of IDDM. Casein has been shown to give a low incidence of diabetes when it is the sole protein source in a weaning diet (Hoorfar et al., 1991; Hoorfar et al., 1992; Scott et al., 1985; Issa-Chergui et al., 1988). The incidence rates are lower when the casein is given in hydrolyzed form (Hoorfar et al., 1991), although purified non-hydrolyzed casein has also been shown to give a low incidence compared to chow diets (Brogren et al., 1988). Plant proteins have also been examined in relation to the incidence of diabetes in the BB rat. The

most extensively studied have been wheat and soy proteins. Gliadin, the major component of wheat protein (gluten), is the dietary component implicated in celiac disease; an intestinal enteropathic disease which may be autoimmune in nature. This may be significant in the study of IDDM as the incidence of celiac disease is higher in children with IDDM (Gadd et al., 1992). The effect of wheat protein on IDDM incidence in humans has been difficult to study since most infants are exposed to wheat after exposure to many other protein sources and/or at the same time as other food types. In the BB rat, wheat gluten has not been shown to be diabetogenic when included as the protein source in a SP diet, compared to a casein based SP diet (Hoorfar et al., 1991; Elliot & Martin, 1984; Scott & Marliss, 1991). In studies examining the effect of dietary fat on IDDM incidence in the BB rat, very little effect has been shown (Issa-Chergui et al., 1988; Hoorfar et al., 1992).

Soy protein which is the protein source in many infant formulas has been shown to give an intermediate incidence of diabetes in the BBdp rat when compared to casein based diets (Hoorfar et al., 1991; Scott et al., 1994). However it has also been determined that the source of the soy protein is important in determining the disease incidence. Defatted soy flour was found to give an incidence rate of 60% while soy hydrolysate gave a lower incidence rate of 35% (Brogren et al., 1989). This study (Brogren et al., 1989) also supports the theory of a critical period in early life that can influence diabetes development. Comparison between rat pups weaned early (day 23-24) and those weaned later (day 25-27), showed those in the late weaning group had a lower incidence of diabetes.

At the present time the causes for the extreme differences in diabetes incidence attributable to diet have yet to be determined. As in the human disease there are several possibilities. One is that diets which give a low incidence contain a protective component. Two, an autoimmunogenic trigger is contained within the diets giving a high incidence. Currently the latter theory is more widely accepted. The third possibility is that early diet alters the development of the immune system in genetically susceptible individuals or BBdp rats, to either prevent or allow the auto-immune process to take place.

ii.b. The Effect of Diet on Growth

Another possibililty exists that is not related to specific components per se. Chow diets typically give significantly higher rates of growth than SP diets (Elliot & Martin, 1984; Hoorfar et al., 1992). It has been shown that, in a given colony, rats with the highest body weight in the early juvenile period (10-40 days) show a significantly greater risk of developing diabetes later in life (Pederson et al., 1994). The animals that did not develop diaabetes showed lower plasma and pancreatic insulin levels as early as 20 days of age (Pederson et al., 1994). These results may indicate that high growth rates per se, or their effects on pancreatic function, may contribute to their subsequent development of diabetes.

Chapter Two - Research Plan

Rationale

Nutritionally complete weaning diets have been shown to significantly alter diabetes incidence in the Bio-breeding diabetes prone (BBdp) rat (Elliot & Martin, 1984; Scott et al., 1985). Pups weaned to non-purified (NP) laboratory chow develop diabetes with an incidence rate of 60%-80% (Scott et al., 1994). Pups weaned to a soy or casein based semi-purified (SP) diet have a lower incidence of diabetes, 40%-60% and 20%-30% respectively (Scott et al., 1994). There are several possibilities to explain these differences. The first is that NP chow diets contain some antigenic trigger which initiates the auto-immune response. It is also possible that weaning to nutritionally different diets alters the development of the immune system in general which may lead to alterations in potential autoimmune responses. Chow diets also lead to an increased rate of growth compared to SP diets (Elliot & Martin, 1984; Hoorfar et al., 1992) and it is possible that this increased growth rate and its relationship to endocrine function contributes to the disease pathogenesis. Two other theories to explain this difference in incidence rates focus on the SP diets. One is that these diets contain some protective factor which prevents the auto-immune process from occurring. The other is that feeding these diets protects against diabetes by preventing exposure to the auto-immune trigger in NP chow dies.

BBdp rats are known to have irregularities in their immune systems including a pronounced T-cell lymphopenia, particularly in the CD8+ cytotoxic/suppresser subset. Studies to date have focused mainly on incidence rates following weaning to different diets, with very little examination of phenotypic differences in rats fed different diets. As well, most studies using the BB rat begin feeding experimental diets at 21 days of age, the standard age for weaning of rat pups even though pups were exposed to the dams diet which was usually chow. Since changes in immune responses can occur with very little antigen exposure, this brief but early exposure to the dams diet may significantly alter the ontogeny of the pups immune system.

The aim of this study was to examine differences in the immune system of BBdp rats 14 and 30 days of age fed one of three nutritionally complete diets known

to result in different diabetes incidence; NIH chow (high incidence), casein based SP diet (low incidence) or soy based SP diet (intermediate incidence).

Concurrently with the study of the BBdp rats, Bio-Breeding non-diabetic prone (BBn) rats were fed the same diets and served as the control rats in this study.

Hypotheses

1. BBdp rats fed different diets from birth to 30 days of age would show differences in lymphocyte phenotype profiles. Animals fed the chow diet would show the typical BBdp lymphopenic profile. Animals fed the casein based SP diet would show increased numbers of T-cells and those fed the soy based SP diet would be intermediate between the two.

2. Feeding the three diets (NIH chow, casein based SP diet, soy based SP diet) to BBn rats would not significantly alter the phenotypes of lymphocytes.

Research Objectives

The hypotheses will be tested in the following ways.

Hypothesis One

BBdp dams will be fed one of three experimental diets prior to giving birth: commercial NIH-07 chow (meal form), casein-based semi-purified diet or soy-based semi-purified diet. At weaning (21 days of age), pups will be fed the same diet as their respective dams. At 14 and 30 days of age, pups will be killed. At 14 days of age lymphocytes will be isolated from the spleen and immune cell phenotypes will be identified using one way immunofluorescence. At 30 days of age, lymphocytes from spleen, blood and mesenteric lymph nodes will be isolated and immune cell identified by one way immunofluorescence.

Hypothesis Two

The same protocol will be used to examine the effect of the three diets on immune phenotypes in the lymphoid organs of the BBn rats.

Chapter Three - Materials and Methods

i. Chemicals

Bovine serum albumin (fraction V) and trypan blue were purchased from Sigma Chemical Co., St. Louis, MO. Paraformaldehyde was purchased from Anachemia Science, Montreal, PQ, Canada.

ii. Animals and Diets

This study was carried out in accordance with the guidelines of the Canadian Council on Animal Care and was reviewed and approved by the Animal Policy and Welfare Committee, in the Faculty of Agriculture and Forestry.

Male and female BBn and BBdp animals were obtained and from the Department of Agricultural, Food and Nutritional Sciences colony. The original breeding stock for this colony was obtained from the Animal Resources Division, Health Protection Branch, Health Canada, Ottawa ON, Canada.

Three diets were used in this experiment. A non-purified chow diet (NIH-O7 Rodent Diet, Ziegler Bros. Inc. Gardners, PA, USA), and two purified diets with the protein provided as either soy or casein. The two purified diets were made in our laboratory and were based on the AIN-76 diet (ICN, Montreal, PQ, Canada). The lipid content of the purified diets was 42% (w/w) with a P/S ratio of 0.4. These values were based on an average of several infant formulas. The nutrient content of the diets are shown in table 1.

Table 1. Diet Compositions

Casein/Soy[1]			NIH-07[2,3]	
INGREDIENT	%(w/w)		INGREDIENT	%w/w
Carbohydrate (Cornstarch)	27.0		Carbohydrate	49.9
Fat	20.00		Fat	5.7
safflower oil	6.2			
linseed oil	0.72			
beef tallow	13.6			
Casein	27.05		Protein	22.5
Soy protein	27.05			
Non-nutritive Cellulose	8.0		Fibre	3.9

[1] Supplemented (per kg of diet) with;10 g AIN-vitamin mix; 35 g AIN-mineral mix; 3 g DL-methionine and 2 g choline bitartrate. Vitamin mix contains (g/kg mix); thiamin hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride,0.7; nicotinic acid, 3; D-calcium pantothenate, 1.6; folic acid, 0.2; D-biotin, 0.02; Vitamin B_{12} 0.02; D,L-alpha-tocopherol acetate, 20; cholecalciferol, 0.25;sucrase, 973; and 1.2 x 10^5 RE (retinylacetate). Mineral mix contains (mg/kg ix); $CaHPO_4$, 500; NaCl, 74; $K_3C_6H_5O_7 \cdot H20$, 220; K_2SO_4 , 52; MgO, 24; $MnCO_3$, 3.5; $C_6H_5O_7Fe \cdot 3H_2O$, 6; zinc carbonate, 1.6; cupric carbonate, 0.3; KI, 0.01; Na_2SeO_3, 0.01; $CrK(SO_4)_2 \cdot 12H_2O$, 0.55; sucrose, 118.

[2] Supplemented (g/100g) with; NaCl, 0.5; dicalcium phosphate, 1.25; ground limestone, 0.5; premixes, 0.25.

[3] Macronutrients supplied as (g/100g); dried skim milk, 5; fish meal, 10; soybean meal, 12; alfalfa meal, 4; corn gluten meal, 3; ground yellow shelled corn, 24.5; ground hard winter wheat, 23; wheat middlings, 10; Brewer's dried yeast, 2; dried molasses, 1.5; soybean oil, 2.5.

Beginning one week prior to giving birth, dams were randomly assigned to one of the three experimental diets. At birth litters were culled to 8-12 pups. Pups were housed with the dams until 21 d of age when they were weaned onto the same diet as their dam. Pups were fed for 14, or 30 days of age. At these time points they were anaesthetized and killed by cervical dislocation, for study. All animals were fed diet and water ad libitum throughout the study. Food was removed from the cages at 4 pm on the afternoon prior to killing at 30 days of age.

All animals were housed in plastic shoebox cages in a temperature and humidity controlled facility, maintained on a 12 h light/dark cycle. Upon weaning cage inserts were placed in the bottom of the cages to limit coprophagia. Weanling rats were housed in groups of 2-4 animals per cage.

iii. Lymphocyte Isolation

a) Blood

Animals were anesthetized by halothane. A cardiac puncture was performed using a heparinized syringe and needle, to acquire a sample of whole blood, which was aliquoted into chilled 5 ml Vacutainer tubes containing 0.05 ml EDTA (Becton Dickinson, Rutherford NJ). One ml of whole blood was then diluted with 1ml of Hank's Balanced Salt Solution (HBSS) (Ca^+ Mg^+ free) supplemented with O.5% (w/v) BSA. The diluted samples were then placed on 3 ml of 1.119 g/ml Histopaque (Sigma Diagnostics, St.Louis, MO) and centrifuged at 600xg (Jouan centrifuge, Jouan Inc, Winchester, VA) for 25 minutes at 25° C. Lymphocytes were removed from the interface, washed twice in HBSS and assessed for viability using trypan blue. Viability was assessed at greater than 95% for all samples.

b) Spleen, Thymus, Mesenteric Lymph Nodes

Anaesthetized rats (as described above) were killed by cervical dislocation and spleen, thymus and mesenteric lymph nodes (MLN) were removed under sterile conditions. Tissues were placed in chilled sterile petri dishes containing HBSS (as above). All three organs were pressed through nylon mesh (100 μm) to isolate immune cells. Samples were then centrifuged for 10 minutes at 228xg (Beckman J2-HC Centrifuge, Beckman Instruments, Palo Alto, CA), to pellet cells. The splenocytes were then treated with a solution consisting of 155 mmol/L NH_4Cl,

0.1 mmol/L disodium EDTA and 10 mmol/L $KHCO_3$ to lyse red blood cells and washed twice with HBSS. Lymphocytes were assessed for viability using trypan blue. Viability was assessed at greater than 95% for all samples.

iv. Mononuclear cell phenotyping

Lymphocyte subsets from spleen and thymus (day 14) and from spleen, blood and mesenteric lymph nodes (day 30) were characterized by immunofluorescence assay, using supernatants from hybridoma-secreting mouse monoclonal antiodies specific for different rat monoclonal cell subsets. (Unless otherwise indicated, antibodies were kindly provided by Dr. A. Rabinovitch, Edmonton AB, Canada). All antibodies were mouse anti-rat IgG. Table 2 provides specificity of the monoclonal antibodies.

Table 2. Monoclonal Antibody Specificity

Antibody	Determinant	Cell Recognition
OX19	- CD5	- thymocytes - T-lymphocytes
W3/25	- CD4	- T helper cells
OX8	- CD8	- T suppressor/cytoxic cells - Natural Killer cells
OX12	- kappa chain of surface immunoglobulin	- B lymphocytes
3-2-3	- triggering structure similar to CD16	- Natural Killer cells
OX42	- C3bi complement receptor	- macrophages, monocytes granulocytes
OX33	- CD45RA	- B cells
W3/13	- thymocyte glycoprotein	- thymocytes, T cells, stem cells, plasma cells, polymorphs

Aliquots of 2-5 x 10^5 cells from all four cell groups were incubated for 30 min at 4° C with each antibody (with the except for cells from thymus and mesenteric lymph nodes which were not incubated with OX42 or 323). Cells were then washed three times with 200 µl of PBS supplemented with fetal calf serum (40 g/100L) and incubated for another 30 min with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (FITC). To determine background fluorescence due to non-specific binding of the FITC antibody, an aliquot of each cell group was incubated for 30 minutes with the FITC antibody alone. The cells were then washed three times (as above) and fixed in PBS containing 1% w/v paraformaldehyde. Relative fluorescence intensity for each antibody was determined by analysis using a FACScan (Becton Dickinson, Sunnyvale, CA). The resulting percentages were corrected for background fluorescence using the analysis of the cells incubated with FITC alone.

vii. *Statistical Analysis*

Mean ± SEM were analyzed by one-way ANOVA. The significance level was set at $p<0.05$ and differences between groups were identified by the Duncan multiple-range test . Effect of diet on age differences was determined using the general linear model procedure and estimate command. All statistical analysis was conducted using the SAS statistical package (Version 6, SAS Institute, Cary, NC).

Chapter Four - BBdp Results

BBdp Animal Characteristics

Day 14 (Table 4.1)

At 14 days of age there were differences in body weights, with the NIH group being the heaviest (29.7 ± 0.6) and significantly heavier than the SOY group (24.2 ± 2.1). However values for spleen weight (g)/body weight (g) and thymus weight (g)/body weight (g) did not differ. Spleen weight, total splenocytes, splenocytes/g body weight and thymus weight were also not significantly different.

Splenocytes/g spleen differed significantly between groups with the NIH group having significantly ($p<0.01$) more than the CAS or SOY groups. As well the total number of thymocytes and thymocytes/g thymus differed significantly ($p<0.01$) between groups with the NIH group having the highest value in both cases and the SOY group the lowest. With respect to the total number of thymocytes the CAS group did not differ from the other two. In the case of thymocytes/g thymus the CAS group did not differ from the NIH group but had a value significantly higher than the SOY group.

Day 30 (Table 4.2)

At 30 days of age the body weights of the three groups were significantly different ($p<0.05$) with the NIH fed group 10% heavier than the two SP diet groups which did not differ from each other. As well spleen weights differed between groups with the CAS group weighing the most and the SOY group the least ($p<0.05$), the NIH group did not differ from either. Corresponding to this there was a significant difference in the ratios of spleen weight to body weight with the CAS fed animals having significantly ($p<0.05$) heavier spleens for their body weight than the other two groups.

Table 4.1 Day 14 Animal Characterstics - BBdp[1]

	Diet			Minimum Level of Significance[2]
	NIH n = 7	CAS n = 10	SOY n = 5	
Body Weight (g)	29.7 ± 0.6[a]	26.4 ±1.1[ab]	24.2 ± 2.1[b]	0.01
Spleen Weight (mg)	169 ± 10	161 ± 15	180 ± 40	NS
# Splenocytes ($x10^6$)	40.4 ± 5.0	29.4 ± 4.0	31.5 ± 7.0	NS
# Splenocytes($x10^6$) / g spleen	234.4 ± 19.6[a]	178.0 ± 10.7[b]	172.0 ± 9.5[b]	0.01
# Splenocytes ($x10^6$) / g body wt	1.4 ± 0.2	1.1 ± 0.09	1.2 ± 0.2	NS
Spleen wt (g) / body wt (g)	0.0057 ± 0.0003	0.0060 ± 0.0002	0.0068 ± 0.0009	NS
Thymus Weight (mg)	122 ± 8	113 ± 7	98 ± 13	NS
# Thymocytes ($x10^6$)	236 ± 27.8[a]	201 ± 17.7[ab]	132.9 ± 26.3[b]	0.01
# Thymocytes ($x10^6$)/ g thymus	1898 ± 148[a]	1760 ± 86[a]	1293 ± 129[b]	0.01

[1] Values represent mean ± SEM

[2] Significance determined by 1-way ANOVA, NS = P>0.05. Values with different superscripts are significantly different.

Table 4.2 Day 30 Basic Data - BBdp[1]

	Diet			Minimum Level of Significance[2]
	NIH	CAS	SOY	
Body Weight (g)	89.0 ± 3.7[a]	80.5 ± 2.6[b]	80.4 ±3.1[b]	0.05
Spleen Weight (mg)	378 ± 20[ab]	415 ± 30[a]	337 ± 10[b]	0.05
# Splenocytes ($x10^6$)	96.6 ± 8.3	100.6 ± 13.1	72.8 ± 4.1	NS
# Splenocytes($x10^6$) / g spleen	253 ± 15	240 ± 23	222 ± 16	NS
# Splenocytes ($x10^6$) / g body wt	1.1 ± 0.07	1.2 ± 0.16	0.9 ± 0.07	NS
Spleen wt (g) / body wt (g)	0.0043 ± 0.0001[b]	0.0051 ± 0.0003[a]	0.0042 ± 0.0002[b]	0.02

[1] Values represent mean ± SEM

[2] Significance determined by 1-way ANOVA, NS = P>0.05. Values with different superscripts are significantly different.

BBdp Phenotypes

Day 14 Splenocyte phenotypes

Feeding the dams different diets led to differences in splenocyte phenotypes at 14 days of age. The pups from the NIH fed group had a significantly lower percentage of $OX19^+$ lymphocytes than the CAS group ($p<0.05$) while the SOY group was not different from the other two groups (Fig. 4.1.1). With the W3/13 antibody, the SOY group had a significantly lower proportion of $W3/13^+$ lymphocytes than the other two groups ($p<0.05$) (Fig. 4.1.2). When presented as a ratio of $OX19^+$ cells to $W3/13^+$ cells (Fig. 4.1.3) the NIH group's ratio was significantly lower by almost 50% ($p<0.05$) compared to the other two. There were no significant differences between groups in either the $CD4^+$ or $CD8^+$ subsets or the CD4/CD8 ratios (Fig. 4.1.4, Fig. 4.1.5, Fig. 4.1.6).

Both B cell markers OX12 (Fig. 4.1.7) and OX33 (Fig. 4.1.8) showed the same trend, with the NIH fed group having a significantly lower percentage of positive lymphocytes than the other two groups (OX12 $p<0.04$, OX33 $p<0.05$). No differences were seen between any groups when analyzed with the 3·2·3 (NK cells) (Fig 4.1.9) or OX42 (macrophages) (Fig. 4.1.10) markers.

Day 30 Splenocyte Phenotypes

At 30 days of age the NIH fed group had a significantly lower $OX19^+$ percentage (Fig. 4.2.1) than either the CAS or SOY SP diet groups ($p<0.01$). There were no differences in the relative percentages of $W3/13^+$ cells (Fig.4.2.2), however the lower percentage of $OX19^+$ cells led to a significantly lower ratio of $OX19^+$ to $W3/13^+$ (Fig. 4.2.3) cells in the NIH group ($p<0.05$).

In both the $CD4^+$ (W3/25) and $CD8^+$ (OX8) subgroups of T cells (Figs. 4.2.4 & 4.2.5), the same trend was seen with the NIH group having a lower percentage of positive splenocytes in both of these subsets ($p<0.05$). There was no significant difference in the CD4/CD8 ratio between two groups (Fig. 4.2.6).

With the B cell markers OX12 (Fig. 4.2.7) and OX33 (Fig. 4.2.8) the CAS group had a significantly lower percentage of positive cells than the SOY group with both anti-bodies (OX12 $p<0.03$, OX33 $p<0.002$) and was also lower than the NIH group for the percentage of cells positive for OX33 ($p<0.02$). There were no

differences between the NIH and CAS groups with the OX12 anti-body and no differences between NIH and SOY groups with the OX33 anti-body.

The CAS diet group had a significantly higher proportion of 323^+ (NK) (Fig. 4.2.9) cells than either of the other two groups ($p<003$) and the NIH fed group had a significantly lower proportion of $OX42^+$ (macrophage) (Fig. 4.2.10) cells than either the CAS or SOY groups ($p<0.0001$).

Day 30 Mesenteric Lymph Node Phenotypes

In lymphocytes isolated from the MLN, differences were seen in all T cell groups. The NIH fed group had significantly lower percentage of $OX19^+$ cells (Fig. 4.3.1) than either of the SP diet groups ($p<0.03$) which did not differ significantly with respect to this anti-body. Analysis with the W3/13 anti-bodies (Fig. 4.3.2) showed the CAS fed group having a significantly higher percentage of positive lymphocytes than either the NIH or SOY diet groups. The SOY group had a significantly higher ratio of $OX19^+/W313^+$ (Fig. 4.3.3) cells compared to the other two groups ($p<0.001$).

With both the CD4 and CD8 T cell subset markers (Figs. 4.3.4 & 4.3.5), the NIH fed group had the lowest percentages of positive cells, with this difference being significant when compared to the CAS diet group the CD4 (W3/25) anti-body ($p<0.02$) and the SOY diet group for the CD8 (OX8) anti-body ($p<0.01$). The NIH group had a significantly higher CD4/CD8 ratio (Fig. 4.3.6) compared to the two SP diet groups ($p<0.01$). There were no differences between diet groups with either the OX33 (Fig. 4.3.7) and OX12 (Fig. 4.3.8).

Blood Day 30

Significant differences were seen in blood lymphocyte phenotypes between diet groups with all anti-bodies. The NIH fed group had a significantly lower ($p<0.02$) percentage of $OX19^+$ cells than either of the SP group (Fig. 4.4.1) and both the NIH and SOY fed groups had significantly lower ($p<0.0001$) $W3/13^+$ lymphocyte proportions than the CAS group (Fig.4.4.2). All three groups were significantly different from each other ($p<0.05$) with respect to the OX19/W313 ratio, with the SOY fed group having the highest ratio and the CAS the lowest (Fig. 4.4.3).

The same trend was seen with both the $CD4^+$ ($W3/25^+$) and $CD8^+$ ($OX8^+$) cells (Figs. 4.4.4 & 4.4.5). With both markers the NIH fed group had a lower percentage of positive cells ($CD4^+$ $p<0.02$, $CD8^+$ $p<0.03$). The very low percentage of $CD8^+$ cells in the NIH fed group led to a significantly higher CD4/CD8 ratio ($p<0.0001$) in this group, compared to the other two (Fig. 4.4.6).

The SOY fed group had significantly higher B lymphocyte percentages than either the NIH or CAS groups which didn't differ from each other (Figs. 4.4.7 &4.4.8). This was the case for both the OX12 ($p<0.02$) and OX33 ($p<0.0001$) anti-bodies. The CAS fed group had a significantly higher percentage of both 323^+ (NK) cells ($p<0.006$) and $OX42^+$ (macrophages) cells ($p<0.0009$) than either the NIH or SOY groups which did not differ from each other (Figs. 4.4.9 & 4.4.10).

Day 14 Splenocyte Pheontypes

Figure 1.2.1 OX19[1,2]

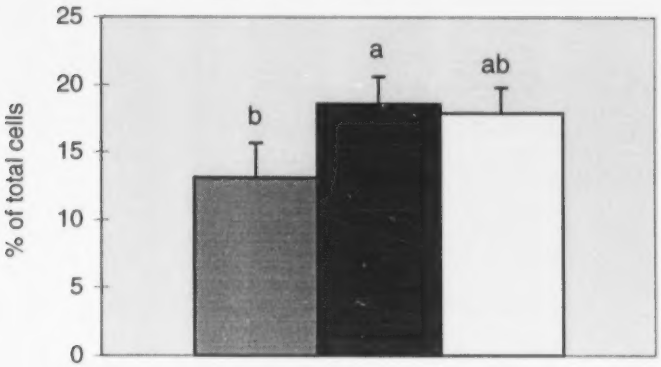


Figure 1.2.2 W3/13[1,2]

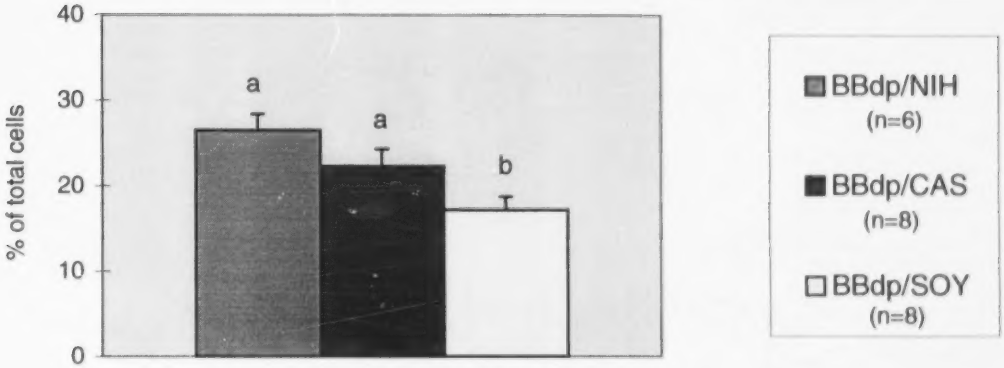
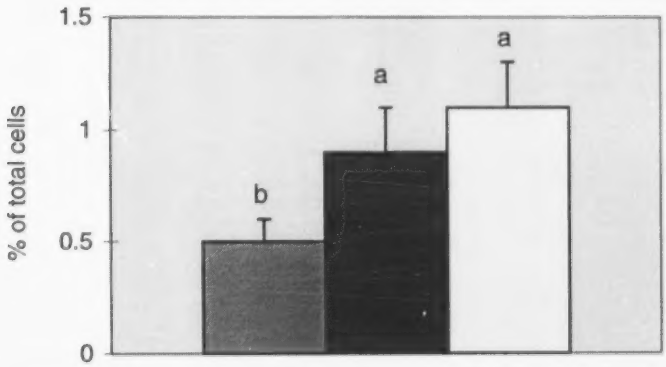


Figure 1.2.3 OX19/W313 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p > 0.05$

Day 14 Splenocyte Phenotypes - (cont)

Figure 1.2.4 W3/25 (CD4)[1,2]

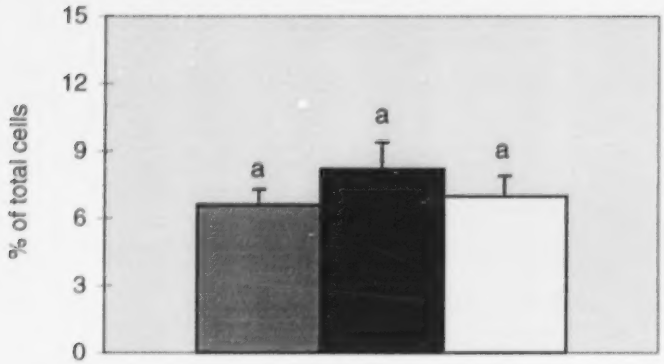
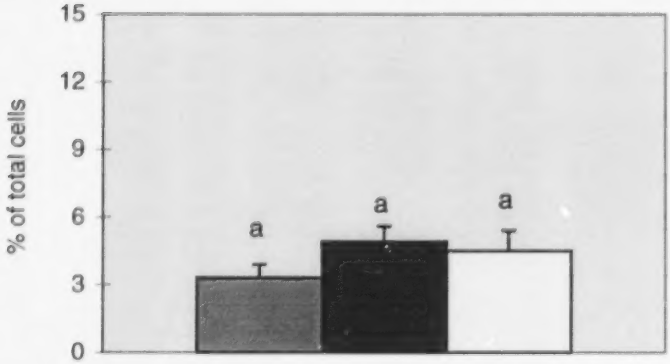


Figure 1.2.5 OX8 (CD8)[1,2]



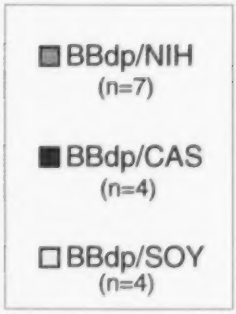


Figure 1.2.6 CD4/CD8 ratio[1,2]

[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p > 0.05$

Day 14 Splenocyte Phenotypes - (cont)

Figure 1.2.7 OX12[1,2]

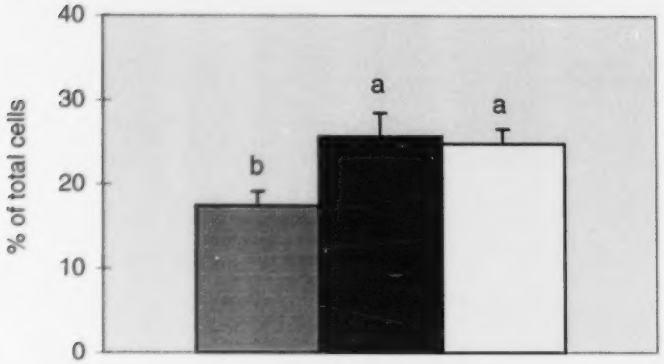


Figure 1.2.8 OX33[1,2]

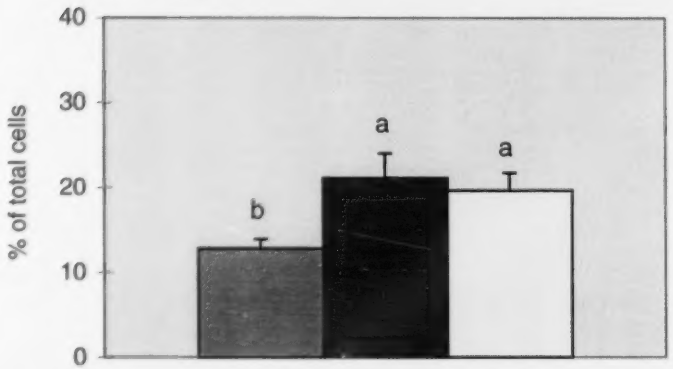
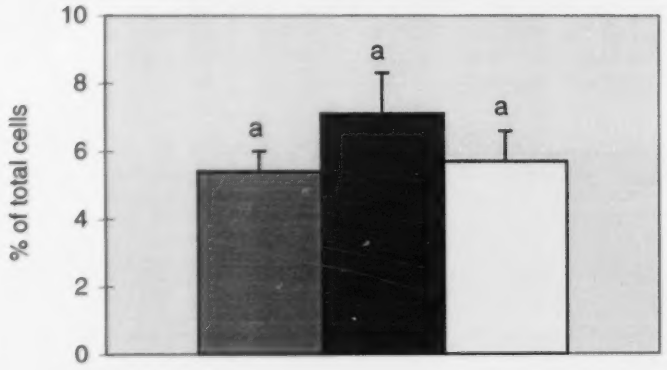


Figure 1.2.9 3·2·3[1,2]

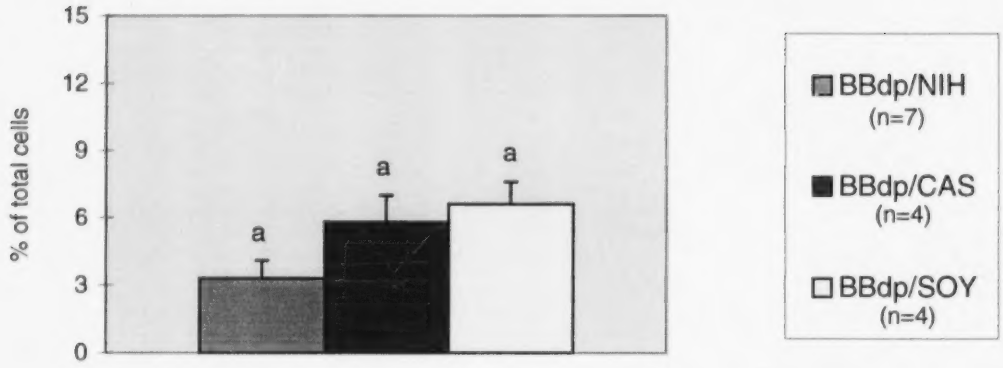


[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p > 0.05$

Day 14 Splenocyte Phenotypes - (cont)

Figure 1.2.10 OX42[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p > 0.05$

BBdp Day 30 Splenocyte Phenotypes

Figure 4.2.1 OX19[1,2]

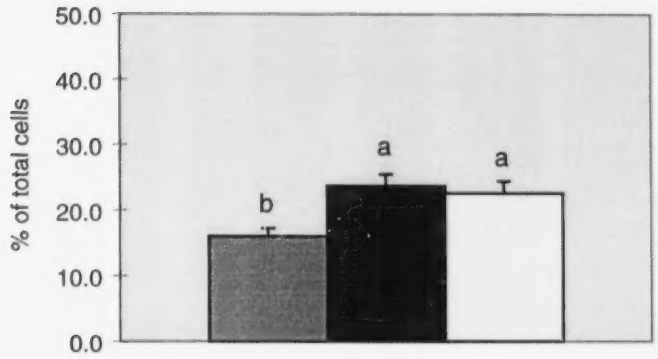


Figure 4.2.2 W3/13[1,2]

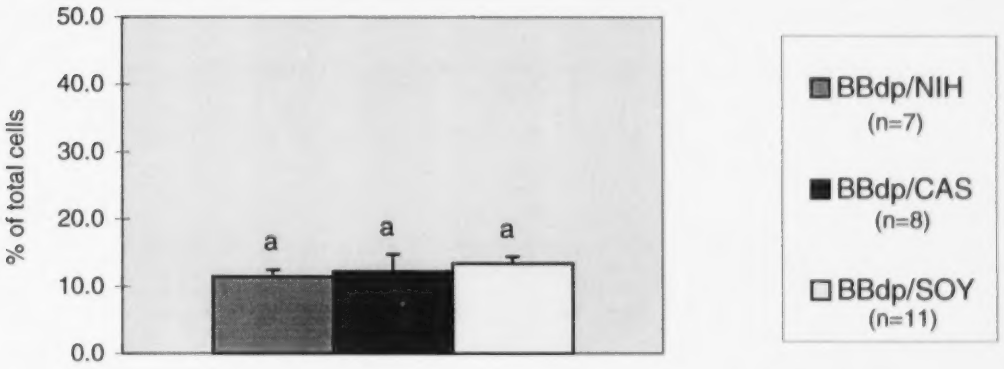
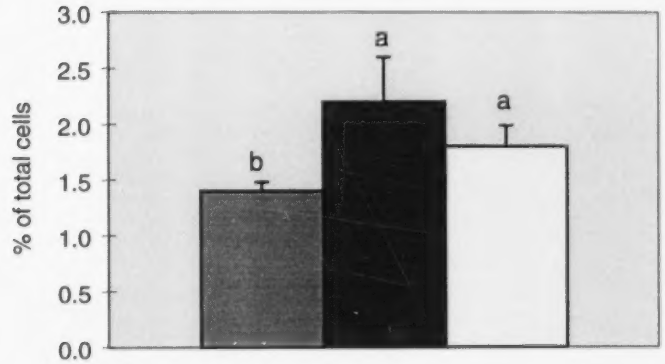


Figure 4.2.3 OX19/W313 ratio[1,2]



[1] Graph represents mean ±SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significanly different. NS = p>0.05

BBdp Day 30 Splenocyte Phenotypes - (cont.)

Figure 4.2.4 W3/25 (CD4)[1,2]

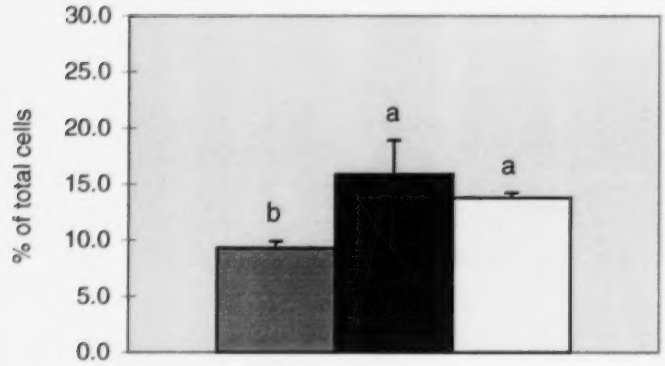
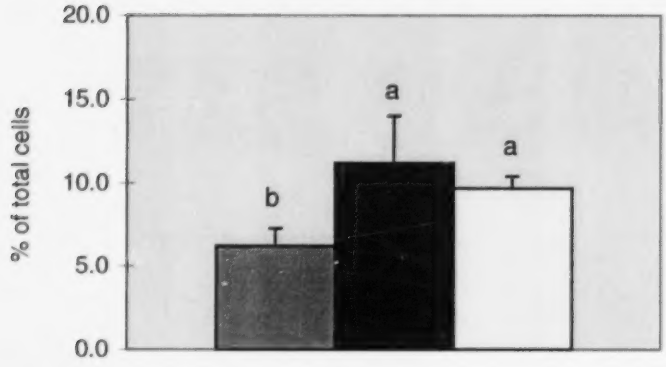


Figure 4.2.5 OX8 (CD8)[1,2]



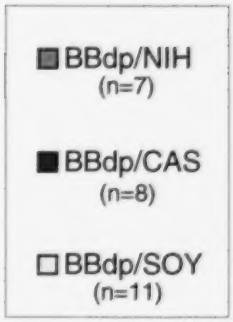
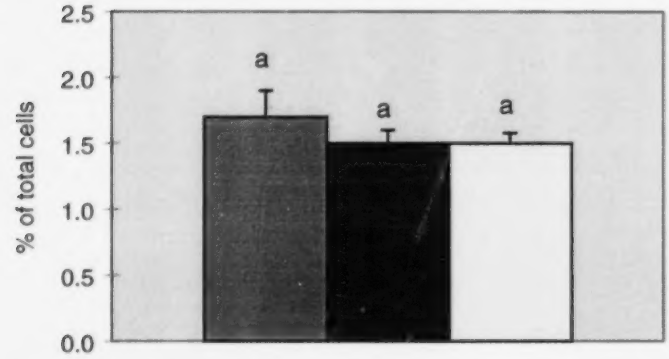


Figure 4.2.6 CD4/CD8 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBdp Day 30 Splenocyte Phenotypes - (cont)

Figure 4.2.7 OX12[1,2]

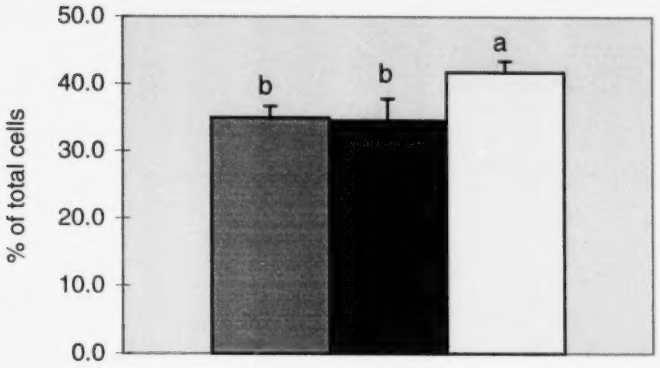
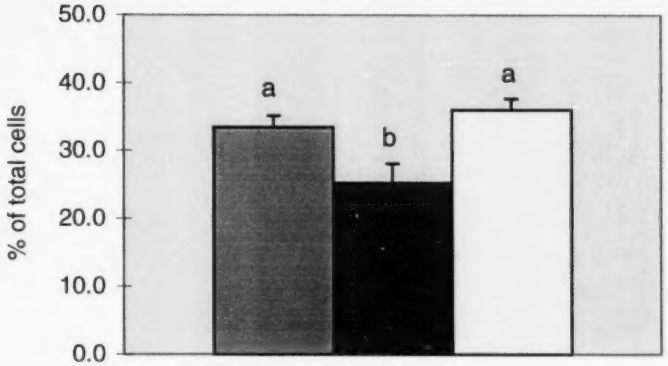


Figure 4.2.8 OX33[1,2]



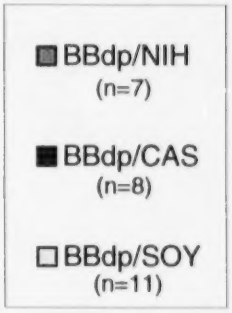
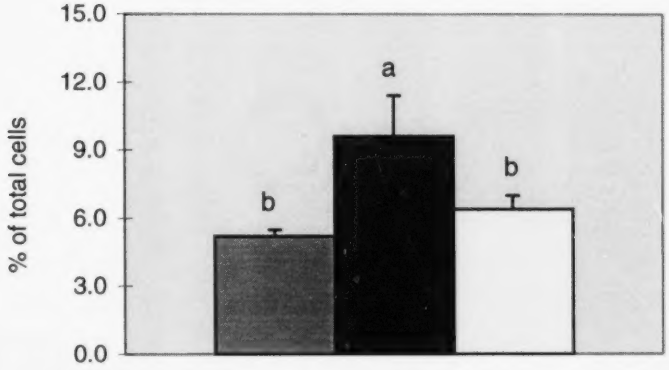


Figure 4.2.9 3·2·3[1,2]

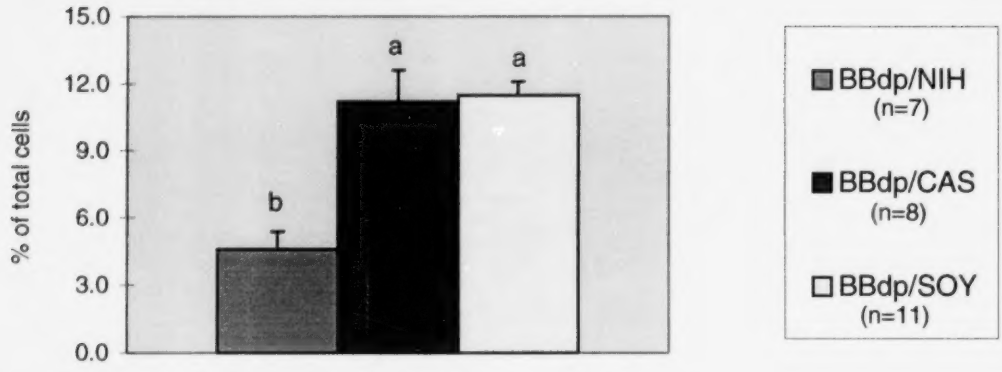


[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBdp Day 30 Splenocyte Phenotypes - (cont.)

Figure 4.2.10 OX42[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBdp Day 30 Mesenteric Lymphocyte Phenotypes

Figure 4.3.1 OX19[1,2]

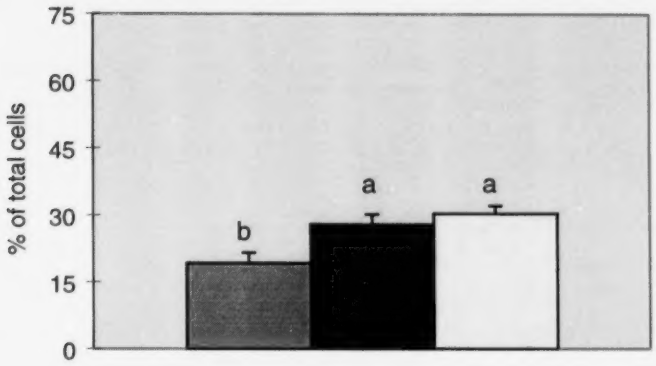


Figure 4.3.2 W3/13[1,2]

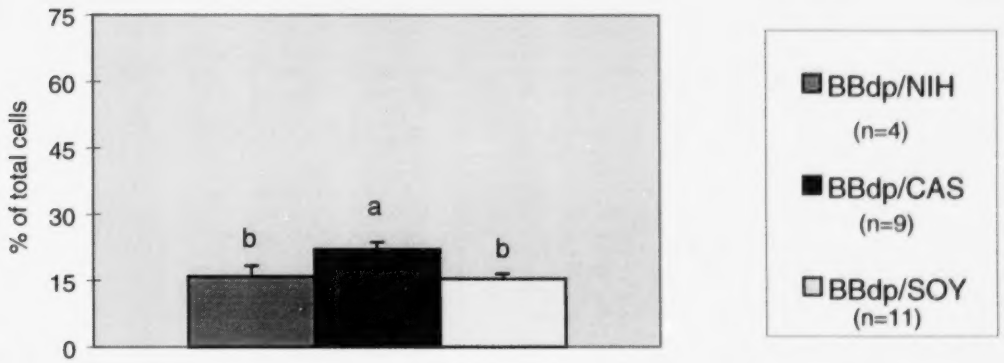
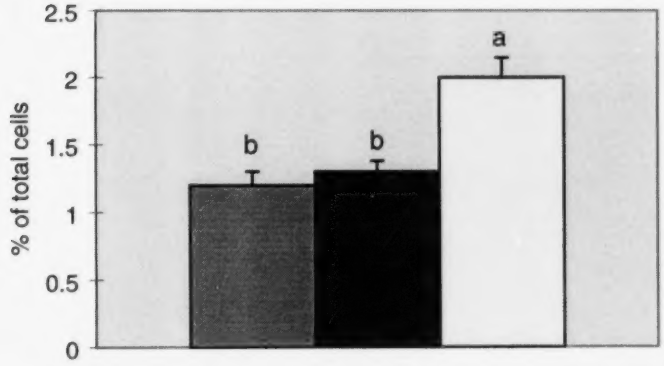


Figure 4.3.3 OX19/W313 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBdp Day 30 Mesenteric Lymphocyte Phenotypes - (cont)

Figure 4.3.4 W3/25 (CD4)[1,2]

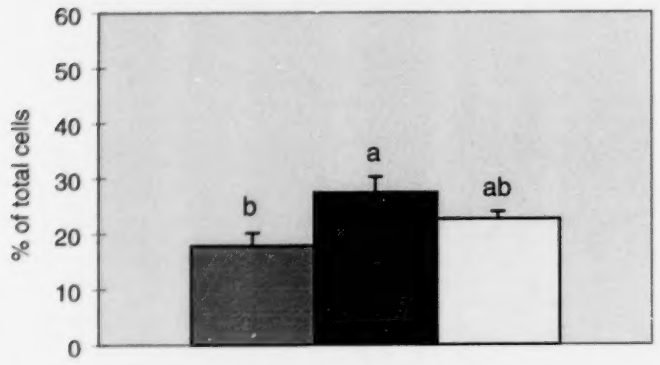


Figure 4.3.5 OX8 (CD8)[1,2]

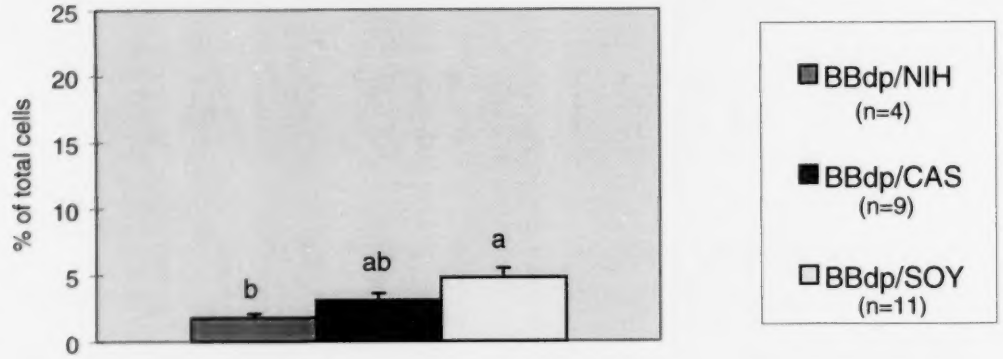
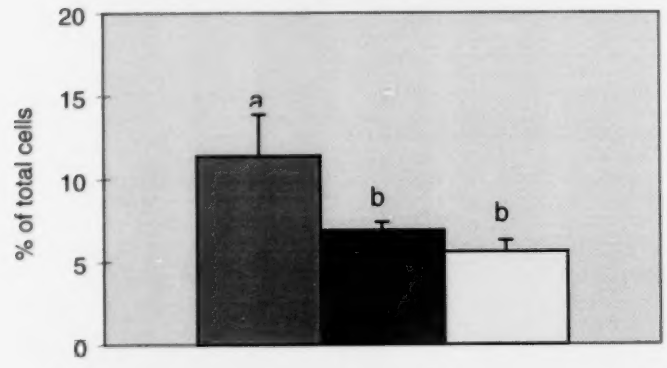


Figure4.3.6 CD4/CD8 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = p>0.05

BBdp Day 30 Mesenteric Lymphocyte Phenotypes - (cont)

Figure 4.3.7 OX12[1,2]

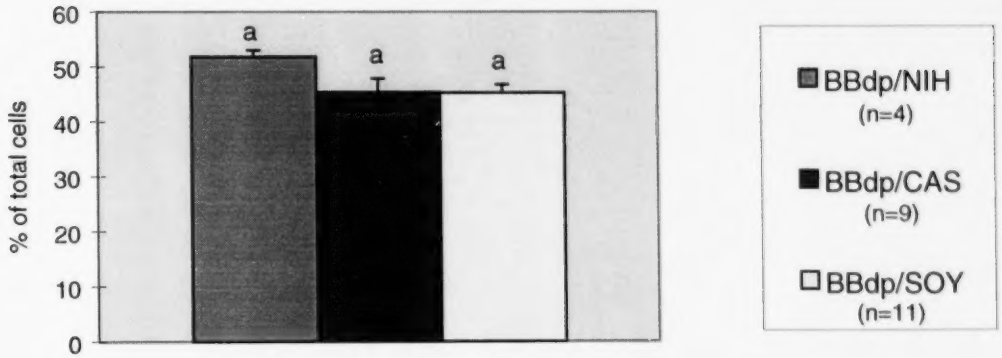
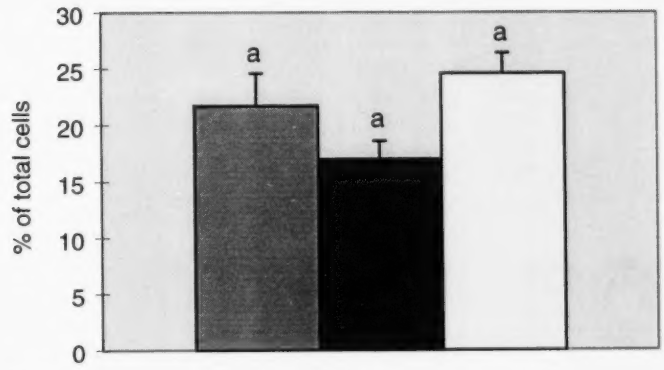


Figure 4.3.8 OX33[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBdp Day 30 Blood Lymphocyte Phenotypes

Figure 4.4.1 OX19[1,2]

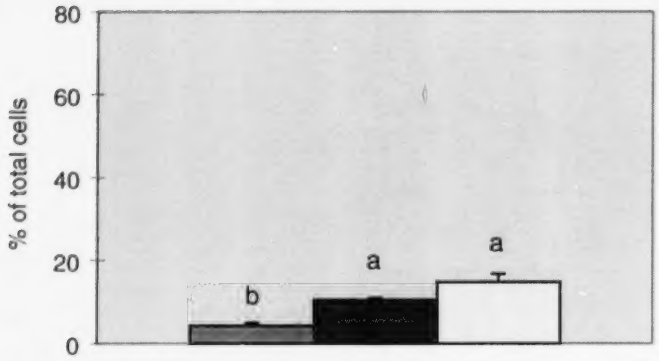


Figure 4.4.2 W3/13[1,2]

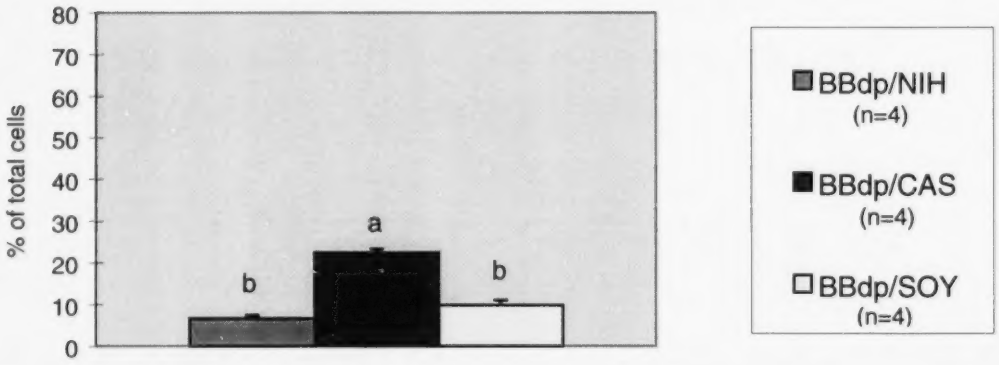
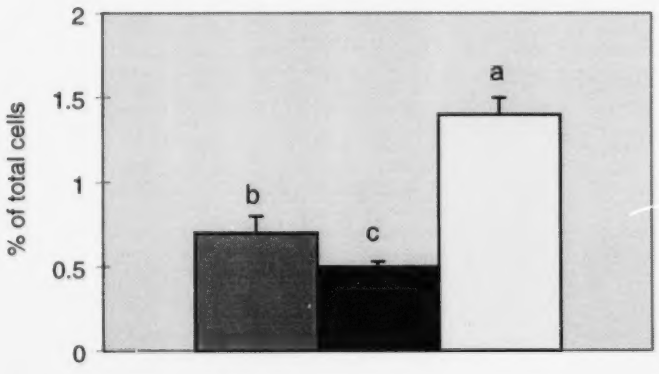


Figure 4.4.3 OX19/W313 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBdp Day 30 Blood Lymphocyte Phenotypes - (cont)

Figure 4.4.4 W3/25 (CD4)[1,2]

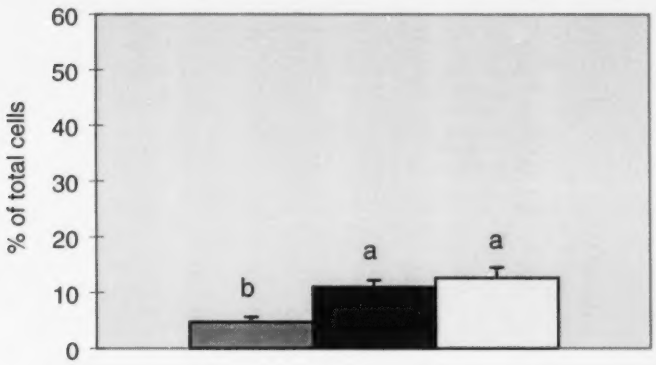
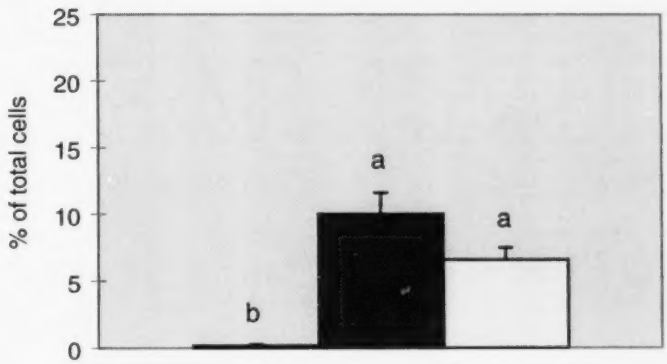


Figure 4.4.5 OX8 (CD8)[1,2]



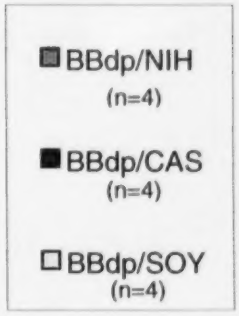
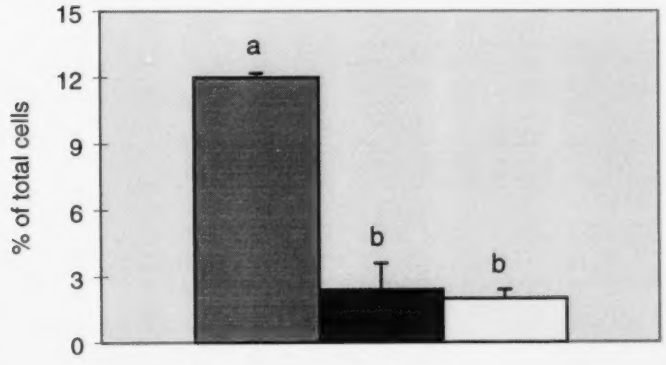


Figure 4.4.6 CD4/CD8 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBdp Day 30 Blood Lymphocyte Phenotypes - (cont.)

Figure 4.4.7 OX12[1,2]

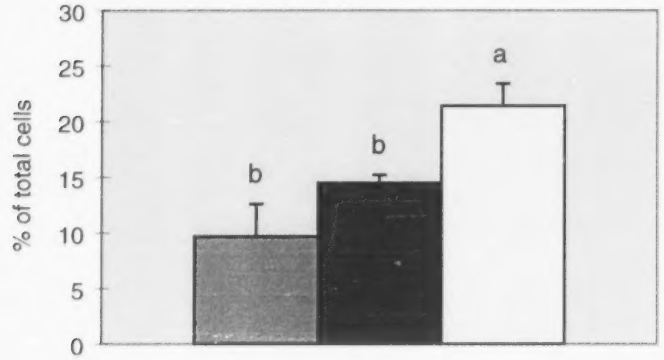


Figure 4.4.8 OX33[1,2]

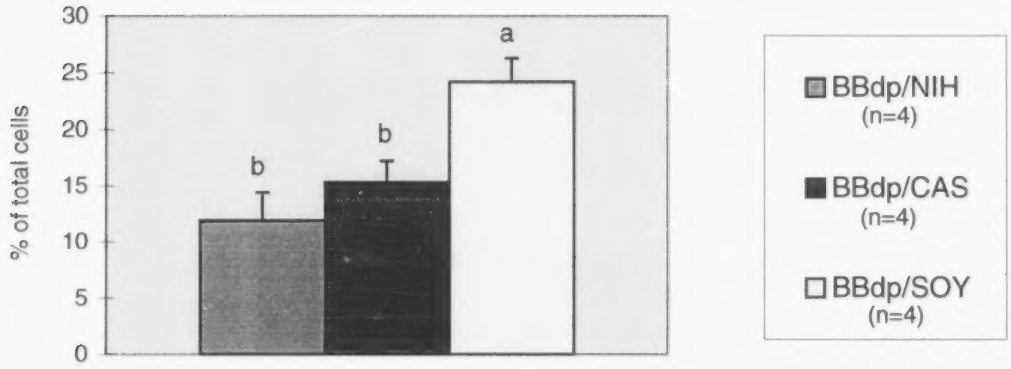
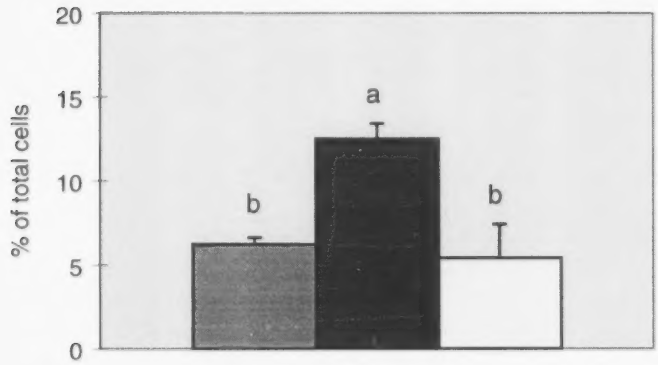


Figure 4.4.9 3·2·3[1,2]

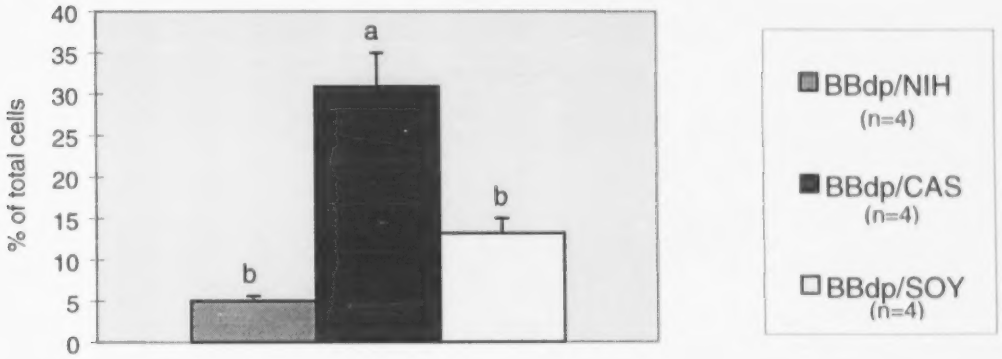


[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBdp Day 30 Blood Lymphocyte Phenotypes - (cont.)

Figure 4.4.10 OX42[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

Effect of Age and Diet on Splenocyte Phenotypes (BBdp)

The effect of age on splenocyte phenotypes was determined for each antibody. The effect of diet on the age differences was only analyzed when at least two diet groups showed changes between ages.

W3/25 (CD4) (Tab. 4.3.1) - All diet groups showed a significant increase in $W3/25^+$ cells between 14 and 30 days of age (NIH $p<0.01$, CAS $p<0.04$, SOY $p<0.0001$). The increase in the CAS group was of a significantly greater magnitude than that of the other two groups ($p<0.05$).

OX8 (CD8) (Tab. 4.3.2) - All diet groups showed a significant increase in $OX8^+$ cells at day 30 compared to day 14 (NIH and CAS $p<0.05$, SOY $p<0.0003$). There was no difference between groups in the magnitude of these changes.

CD4/CD8 ratio (Tab. 4.3.3) - None of the groups showed any significant changes in this ratio between day 14 and 30 days of age.

OX19 (Tab. 4.3.4) - No significant changes in the percentage of $OX19^+$ cells were seen between 14 and 30 days of age.

W3/13 (Tab. 4.3.5) - All three diet groups showed a significant decrease in the proportion of $W3/13^+$ cells at day 30 compared to day 14 (NIH $p<0.0001$, CAS $p<0.02$, SOY $p<0.05$). The NIH group showed a significantly greater change between these two groups than the SOY group ($p<0.05$) while the CAS group did not differ from the other two groups.

OX19/W313 ratio (Tab. 4.3.6) - All three diet groups showed a significant increase in this ratio between 14 and 30 days of age (NIH $p<0.0002$, CAS $p<0.008$, SOY $p<0.03$). There were no differences in the magnitude of these increases.

OX12 (Table 4.3.7) - There were significant increases in the percentage of $OX12^+$ cells in all diet groups between 14 and 30 days of age (NIH and SOY $p<0.0001$, CAS $p<0.05$). The SOY and NIH groups showed a significantly larger increase than the CAS group (p,0.05).

OX33 (Table 4.3.8) - Both the NIH and SOY fed group showed a significant increase in the percentage of $OX33^+$ cells between day 14 and day 30 ($p<0.0001$), however the CAS group showed no significant increase. There was no difference in the magnitude of the changes between the SOY and NIH groups.

3·2·3 (Table 4.3.9) - There were no differences in the proportion of $3{\cdot}2{\cdot}3^+$ cells between 14 and 30 days of age for any of the diet groups.

OX42 (Table 4.3.10) - Both the CAS and SOY groups showed significant increases in their relative numbers of $OX42^+$ cells between day 14 and day 30 ($p<0.01$), however the magnitude of these differences was not different nor were they different from the non-significant changes in the NIH fed group.

Effect of Age and Diet on Splenocyte Phenotypes (BBdp)

Table 4.3.1 W3/25[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	7 ± 0.7	9 ± 0.6	p<0.01	a
CAS	8 ± 1	16 ± 3	p<0.04	b
SOY	7 ± 1	14 ± 0.5	p<0.0001	a

Table 4.3.2 OX8[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	3 ± 0.6	6 ± 1	p<0.05	a
CAS	5 ± 1	11 ± 3	p<0.05	a
SOY	5 ± 1	10 ± 1	p<0.0003	a

Table 4.3.3 CD4/CD8 ratio[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	1.7 ± 0.2	1.7 ± 0.2	NS	N/A
CAS	1.8 ± 0.2	1.5 ± 0.01	NS	N/A
SOY	1.7 ± 0.2	1.5 ± 0.01	NS	N/A

[1] Values represent mean ± SEM

[2] Significance between days within diets was determined by one-way ANOVA, NS = p>0.05

[3] Significance of diets on age differences was determined when at least two diet groups showed changes over time. This was determined by the SAS GLM procedure and the estimate statement. Diet groups that do not share a common letter are significantly different, NS = p<0.05.

Effect of Age and Diet on Splenocyte Phenotypes (BBdp) - (cont.)

Table 4.3.4 OX19[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	13 ± 3	16 ± 1	NS	N/A
CAS	19 ± 2	23 ± 2	NS	N/A
SOY	18 ± 2	23 ± 2	NS	N/A

Table 4.3.5 W3/13[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	26 ± 2	11 ± 1	p<0.0001	a
CAS	22 ± 2	12 ± 2	p<0.02	ab
SOY	17 ± 1	13 ± 1	p<0.05	b

Table 4.3.6 OX19/W313 ratio[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	0.5 ± 0.2	1.4 ± 0.1	p<0.0002	a
CAS	0.9 ± 0.2	2.2 ± 0.4	p<0.008	a
SOY	1.1 ± 0.2	1.8 ± 0.2	p<0.03	a

[1] Values represent mean ± SEM

[2] Significance between days within diets was determined by one-way ANOVA, NS = p>0.05

[3] Significance of diets on age differences was determined when at least two diet groups showed changes over time. This was determined by the SAS GLM procedure and the estimate statement. Diet groups that do not share a common letter are significantly different, NS = p<0.05.

Effect of Age and Diet on Splenocyte Phenotypes (BBdp) - (cont.)

Table 4.3.7 OX12

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	17 ± 2	35 ± 2	p<0.0001	a
CAS	26 ± 3	35 ± 3	p<0.05	b
SOY	25 ± 2	42 ± 2	p<0.0001	a

Table 4.3.8 OX33[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	13 ± 1	34 ± 2	p<0.0001	a
CAS	21 ± 3	25 ± 3	NS	b
SOY	20 ± 2	35 ± 2	p<0.0001	a

Table 4.3.9 3·2·3[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	5 ± 0.5	5 ± 0.3	NS	N/A
CAS	7 ± 1	10 ± 2	NS	N/A
SOY	6 ±1	6 ± 0.6	NS	N/A

[1] Values represent mean ± SEM

[2] Significance between days within diets was determined by one-way ANOVA, NS = p>0.05

[3] Significance of diets on age differences was determined when at least two diet groups showed changes over time. This was determined by the SAS GLM procedure and the estimate statement. Diet groups that do not share a common letter are significantly different, NS = p<0.05.

Effect of Age and Diet on Splenocyte Phenotypes (BBdp) - (cont.)

Table 4.3.10 OX42[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	3 ± 1	4 ± 1	NS	a
CAS	6 ± 1	11 ± 1	$p<0.01$	a
SOY	7 ± 1	11 ± 1	$p<0.01$	a

[1] Values represent mean ± SEM

[2] Significance between days within diets was determined by one-way ANOVA, NS = $p>0.05$

[3] Significance of diets on age differences was determined when at least two diet groups showed changes over time. This was determined by the SAS GLM procedure and the estimate statement. Diet groups that do not share a common letter are significantly different, NS = $p<0.05$.

Chapter Five - BBn Results

BBn Animal Characteristics

Day 14 (Table 5.1)

At 14 days of age there was a significant difference in body weights between the groups with the SOY animals weighing more ($p<0.02$) than animals from the other two groups. Spleen weights between groups did not differ. However total number of splenocytes differed significantly with SOY animals having more than the CAS animals ($p<0.01$) and the NIH animals not differing from either group. As well the ratios of number of splenocytes/g spleen and number of splenocytes/g body weight were significantly different. In both cases the CAS fed group had a significantly lower ratio than the other two groups ($p<0.004$ and $p<0.05$ respectively).

Thymus weight was significantly different between the diet groups however there were no differences when expressed as a ratio with respect to body weight (thymus weight (g)/body weight (g)). As well there were no differences between groups in the ratio of thymocytes/g thymus.

Day 30 (Table 5.2)

At 30 days of age body weights between groups were significantly different ($p<0.0001$) with the NIH fed group weighing more than the two SP diet groups. Spleen weight and the ratios of spleen weight (g)/body weight (g) also differed significantly. The differences in spleen weight followed the same trend as body weight with the NIH group having the heavies spleens and differing significantly from the SOY group ($p<0.01$), the CAS group was not significantly different from the other two. The ratios of spleen weight (g)/body weight (g) also differed significantly with the CAS group having a significantly higher ratio than the other two groups ($p<0.05$).

Total number of splenocytes and splenocytes/g spleen also differed significantly. The CAS group had less splenocytes ($p<0.007$) than the NIH group however the SOY group did not differ from either group. When expressed as a ratio of splenocytes/g spleen the CAS group also had the lowest value and this was significantly lower than the other two groups ($p<0.007$). There was no difference in the number of splenoctyes/g body weight.

Table 5.1 Day 14 - Animal Characteristics (BBn)[1]

	Diet			Minimum Level of Significance[2]
	NIH (n=7)	CAS (n=10)	SOY (n=5)	
Body Weight (g)	24.8 ± 2.1[b]	27.8 ± 1.9[b]	34.8 ± 1.7[a]	p<0.02
Spleen Weight (mg)	158 ± 10	155 ± 20	186 ± 10	NS
# Splenocytes ($x10^6$)	53.4 ± 8.3[ab]	40.3 ± 4.8[b]	67.6 ± 3.8[a]	p<0.01
# Splenocytes($x10^6$) / g spleen	335 ± 19[a]	260 ± 16[b]	364 ± 19[a]	p<0.004
# Splenocytes ($x10^6$) / g body wt	2.1 ± 0.2[a]	1.4 ± 0.1[b]	2.0 ± 0.1[a]	p<0.05
Spleen wt (g) / body wt (g)	0.0062 ± 0.0001[a]	0.0055 ± 0.0002[b]	0.0053 ± 0.0001[b]	p<0.05
Thymus Weight (mg)	121 ± 10[a]	113 ± 10[ab]	98 ± 10[b]	p<0.03
# Thymocytes ($x10^6$)	117 ± 9.9	148 ± 13.7	193 ± 10.5	NS
# Thymocytes ($x10^6$)/ g thymus	1334 ± 58	1471 ± 82	1510 ± 106	NS

[1] Values represent mean ± SEM for each diet group

[2] Significance determined by 1-way ANOVA, NS = P>0.05. Values with different superscripts are significantly different.

Table 5.2 Day 30 - Animal Characteristics (BBn)[1]

	Diet			Minimum Level of Significance[2]
	NIH (n=11)	CAS (n=11)	SOY (n=9)	
Body Weight (g)	91.6 ± 3.6[a]	71.7 ± 3.5[b]	76.0 ± 2.6[b]	p<0.0001
Spleen Weight (mg)	478 ± 20[a]	437 ± 30[ab]	405 ± 20[b]	p<0.01
# Splenocytes ($x10^6$)	194 ± 24[a]	137 ± 16[b]	159 ± 9[ab]	p<0.007
# Splenocytes($x10^6$) / g spleen	402 ± 43[a]	310 ± 22.3[b]	399 ± 25[a]	p<0.02
# Splenocytes ($x10^6$) / g body wt	2.0 ± 0.2	1.9 ± 0.2	2.1 ± 0.1	NS
Spleen wt (g) / body wt (g)	0.0052 ± 0.0005[b]	0.0061 ± 0.0002[a]	0.0053 ± 0.0002[b]	p<0.04

[1] Values represent mean ± SEM for each diet group

[2] Significance determined by 1-way ANOVA, NS = P>0.05. Values with different superscripts are significantly different.

BBn Phenotypes

Spleen Phenotypes Day 14

Feeding the dams different diets produced one significant differences in T cell phenotypes. CAS fed animals had a higher percentage CD8+ cells than the NIH and SOY animals which were also differenct from each other. There was a significant difference in the percentage of B cells that were positive for both the OX12 and the OX33 marker with the CAS fed animals having a higher percentage of positive cells for both antibodies. The SOY group was significantly lower than the other two groups for the percentage of cells positive for the 323 (NK cell). There was no difference between groups in their relative percentages of macrophages

Spleen Phenotypes Day 30

At day 30 several differences could be seen in the spleen phenotypes. There were no differences in the $OX19^+$ cell proportions, however the W3/13 marker showed significant differences between all groups with the CAS group being 50% higher than the NIH group. These differences in $W3/13^+$ values led to significant differences between all groups in the ratio of OX19/W313 cells with the NIH group having a two fold higher ratio compared to the CAS fed group.

The only significant difference seen in the CD4 and CD8 T cell sub-groups was in the SOY fed animals when analyzed for $CD4^+$ cells. This group had a significantly higher proportion of positive than the NIH or CAS groups. Significant differences in the CD4/CD8 ratio were seen between the CAS and SOY groups with the SOY group having a higher ratio.

There were no differences seen with the OX12 or OX33 B cell markers.. No differences were seen between groups in the relative proportions of 323^+ (NK) cells or $OX42^+$ (macrophage) cells.

Mesenteric Lymph Node Phenotypes Day 30

The only diet difference seen in analysis was with OX33 marker which demonstrated a 3 fold higher proportion of positive cells in the NIH fed group compared to the CAS fed group. The NIH fed animals had a higher percentage of $OX33^+$ cells than the SOY fed group, as well.

Blood Phenotypes Day 30

When analyzed for $OX19^+$ Tcells the three diet groups did not differ. However, the NIH group had a smaller proportion of W3/13+ T cells than the other two groups. There was also a difference in the ratio of $OX19^+$ cells to $W3/13^+$ cells with the NIH group having a significantly higher ratio compared to the other two groups. There were no significant differences between either the CD4 or CD8 T cell subsets or in the CD4/CD8 ratio.

Analysis of B cell subsets showed no significant differences between groups for their percentages of $OX12^+$ and $OX33^+$ cells. With this anti-body the NIH group had a significantly higher proportion of positive cells than the CAS group. There were no differences with either the 323 (NK cells) or OX42 (macrophages) markers.

BBn Day 14 Splenocyte Phenotypes

Figure 5.1.1 OX19[1,2]

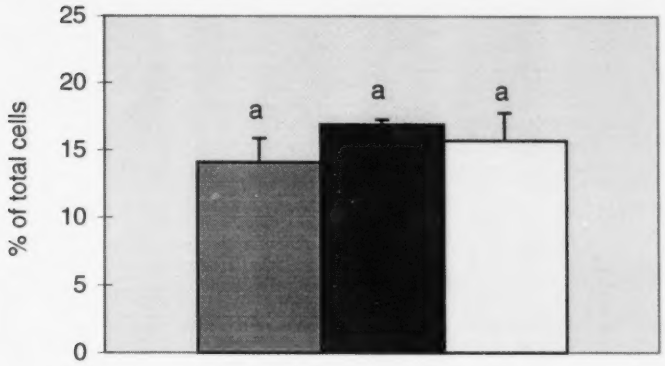


Figure 5.1.2 W3/13[1,2]

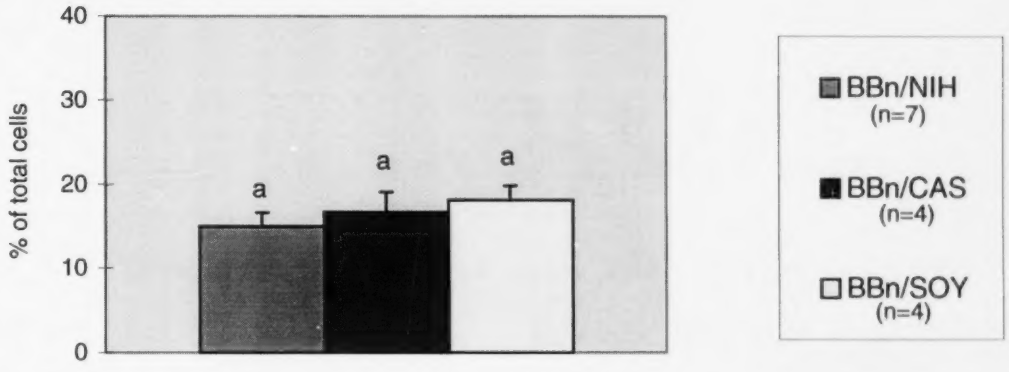
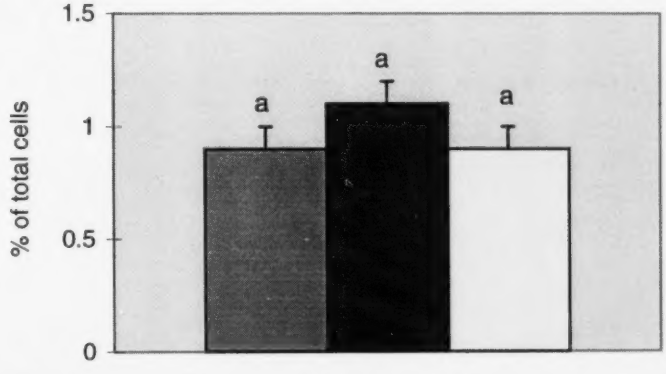


Figure 5.1.3 OX19/W313 (ratio)[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 14 Splenocyte Phenotypes - (cont)

Figure 5.1.4 W3/25 (CD4)[1,2]

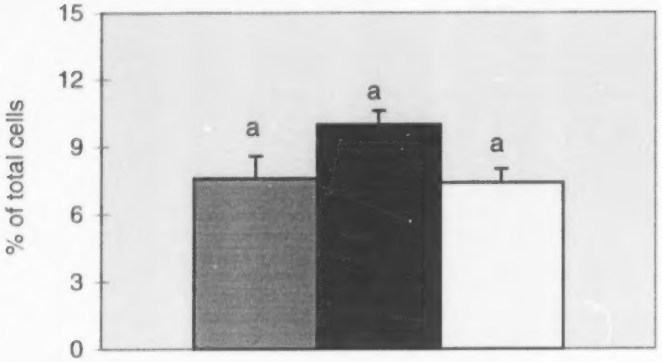


Figure 5.1.5 OX8 (CD8)[1,2]

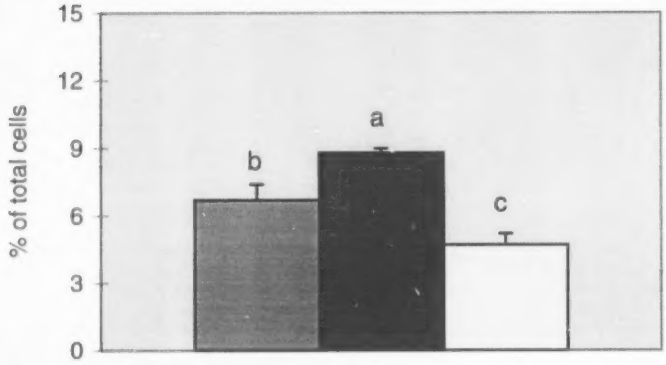
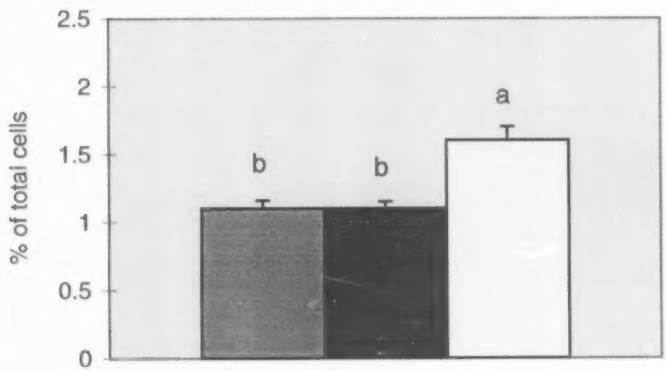


Figure 5.1.6 CD4/CD8 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 14 Splenocyte Phenotypes - (cont)

Figure 5.1.7 OX12[1,2]

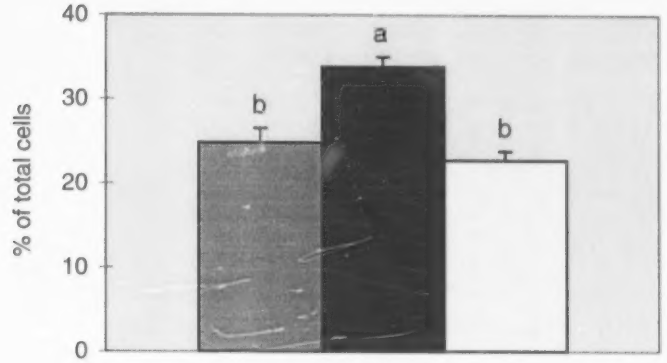


Figure 5.1.8 OX33[1,2]

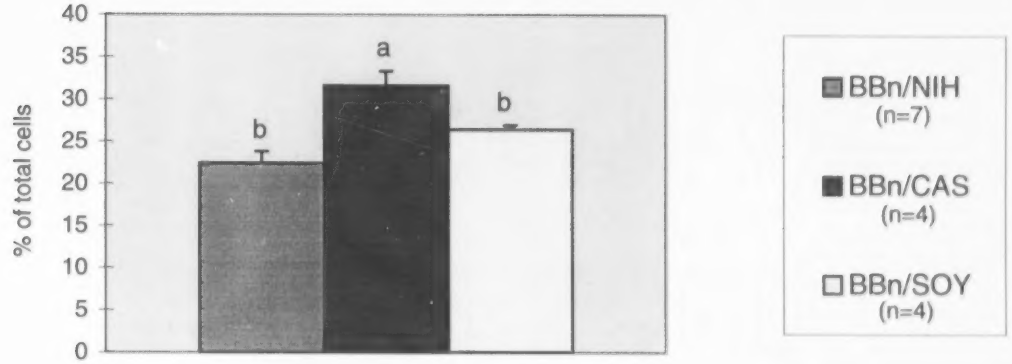
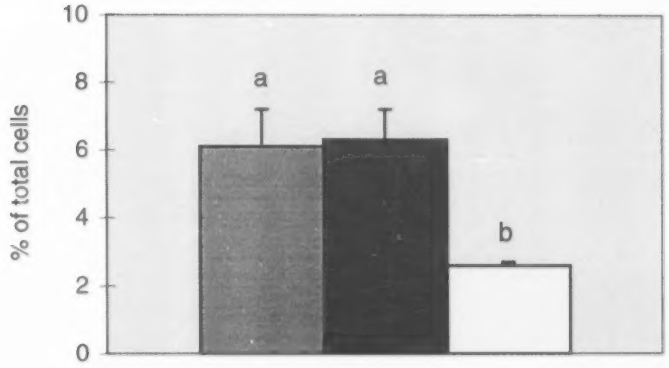


Figure 5.1.9 3·2·3[1,2]

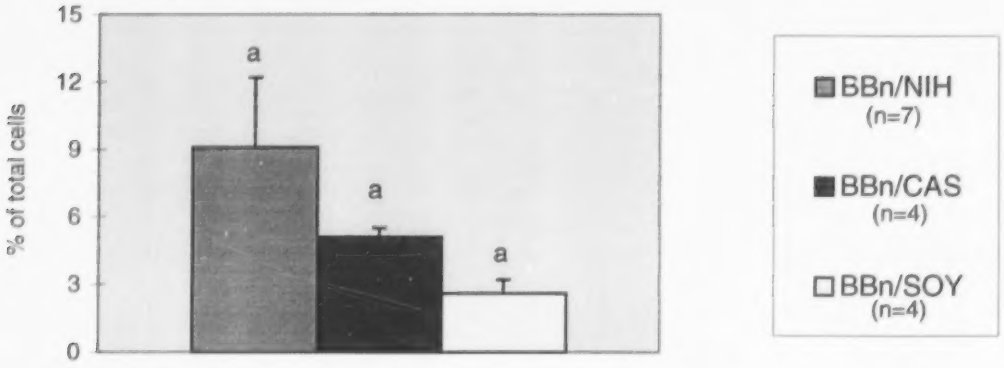


[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 14 Splenocyte Phenotypes - (cont)

Figure 5.1.10 OX42



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 30 Splenocyte Pheontypes

Figure 5.2.1 OX19[1,2]

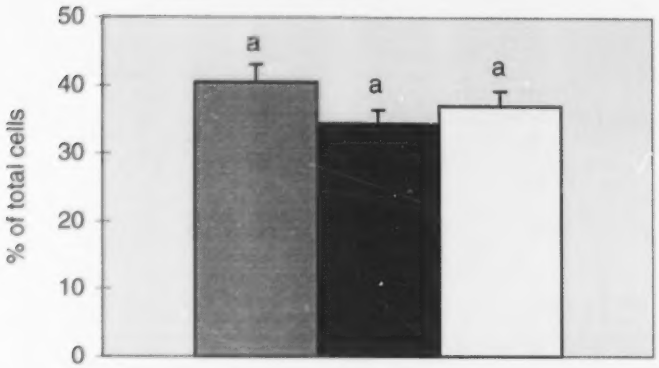


Figure 5.2.2 W3/13[1,2]

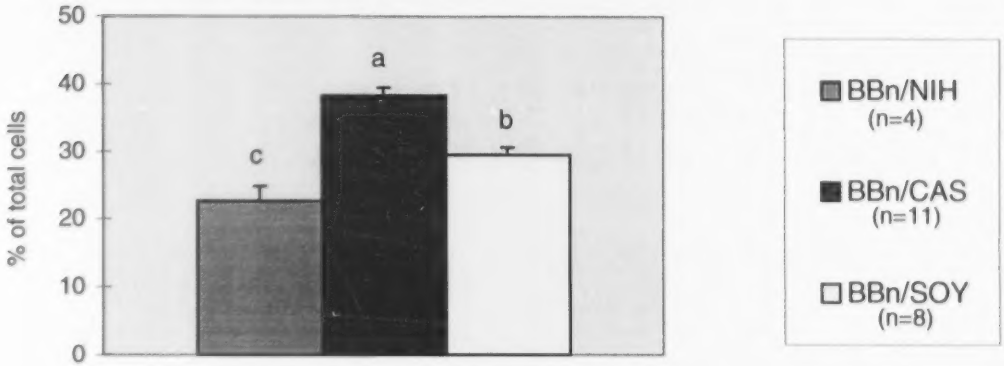
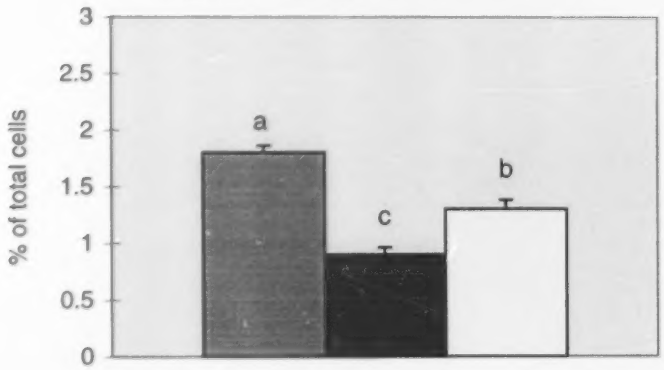


Figure 5.2.3 OX19/W313 ratio[1,2]



[1] Graph represents mean ±SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significanly different. NS = p>0.05

BBn Day 30 Splenocyte Phenotypes - (cont.)

Figure 5.2.4 W3/25 (CD4)[1,2]

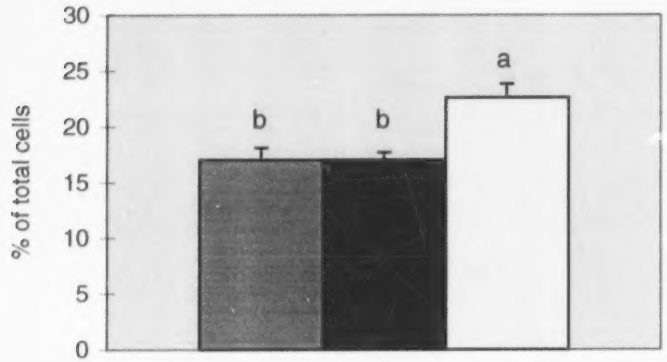


Figure 5.2.5 OX8 (CD8)[1,2]

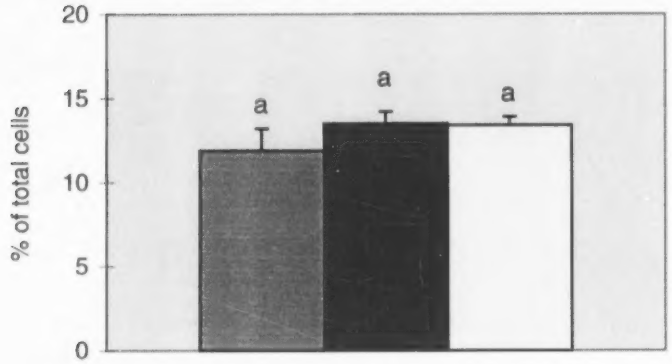
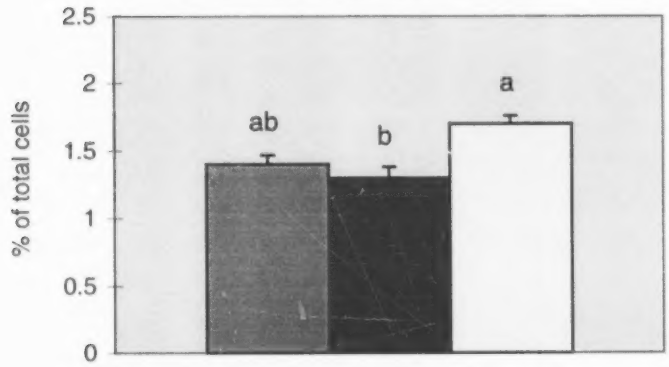


Figure 5.2.6 CD4/CD8 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 30 Splenocyte Phenotypes - (cont)

Figure 5.2.7 OX12[1,2]

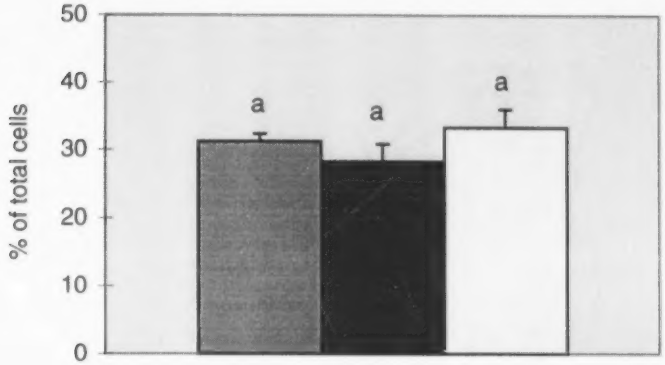


Figure 5.2.8 OX33[1,2]

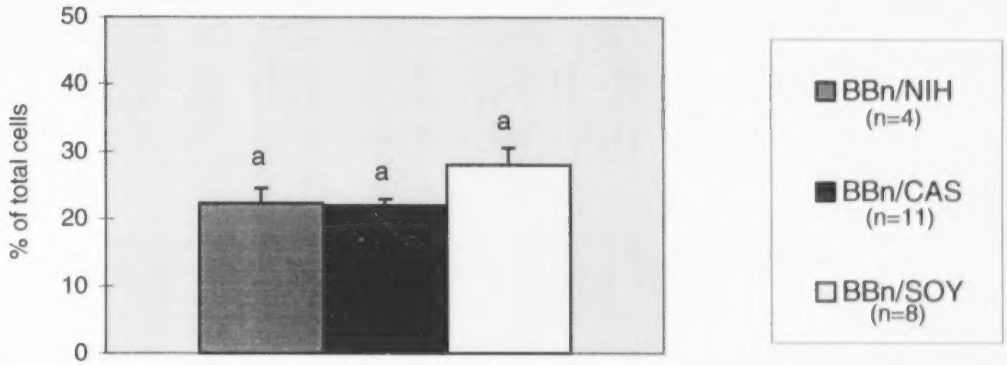
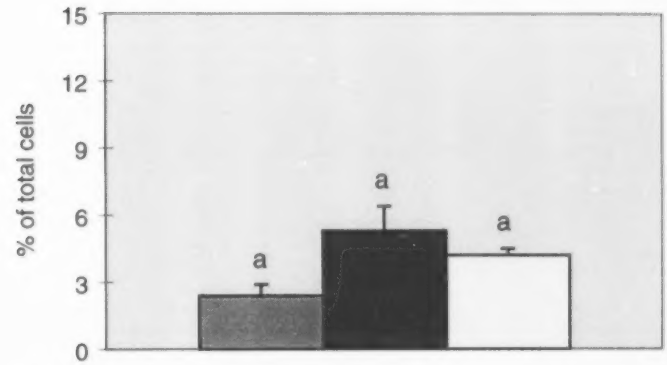


Figure 5.2.9 3·2·3[1,2]

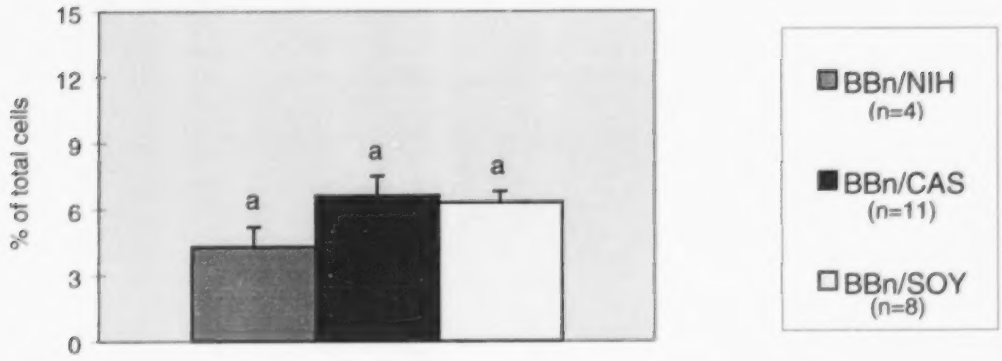


[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 30 Splenocyte Phenotypes - (cont.)

Figure 5.2.10 OX42[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 30 Mesenteric Lymphocyte Phenotypes

Figure 5.3.1 OX19[1,2]

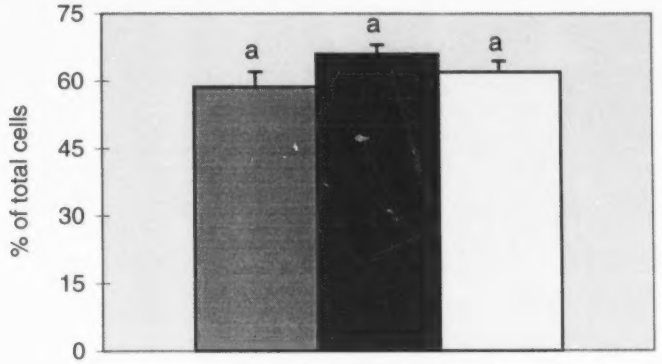


Figure 5.3.2 W3/13[1,2]

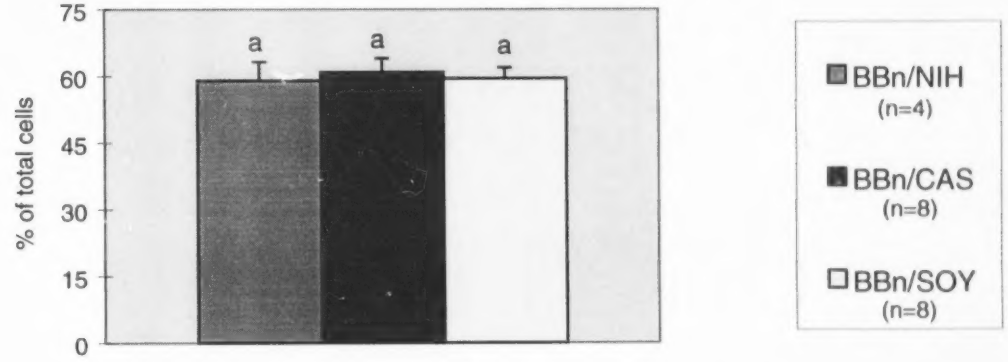
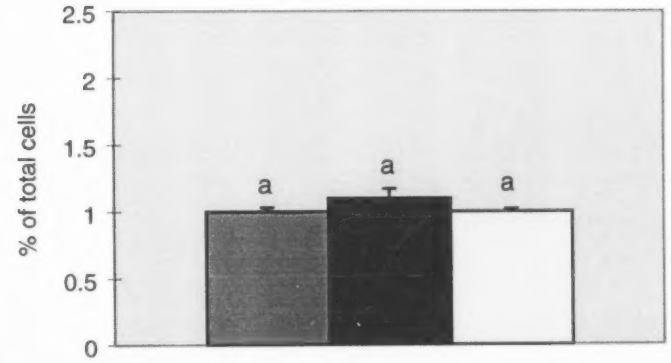


Figure 5.3.3 OX19/W313 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 30 Mesenteric Lymphocyte Phenotypes - (cont)

Figure 5.3.4 W3/25 (CD4)[1,2]

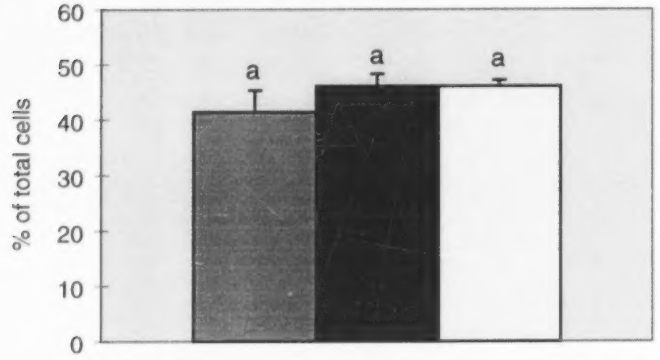


Figure 5.3.5 OX8 (CD8)[1,2]

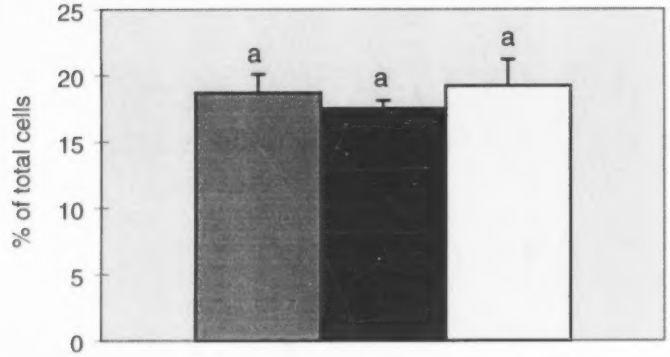
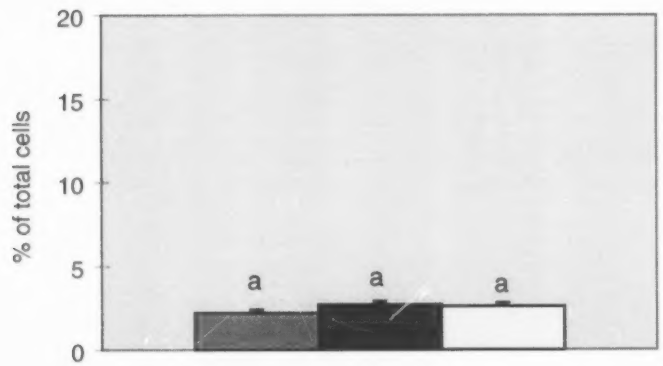


Figure 5.3.7 CD4/CD8 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 30 Mesenteric Lymphocyte Phenotypes - (cont)

Figure 2.6.7 OX12[1,2]

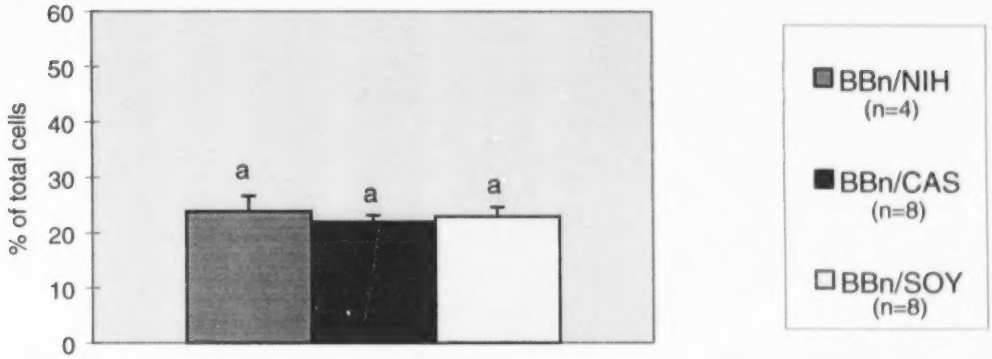
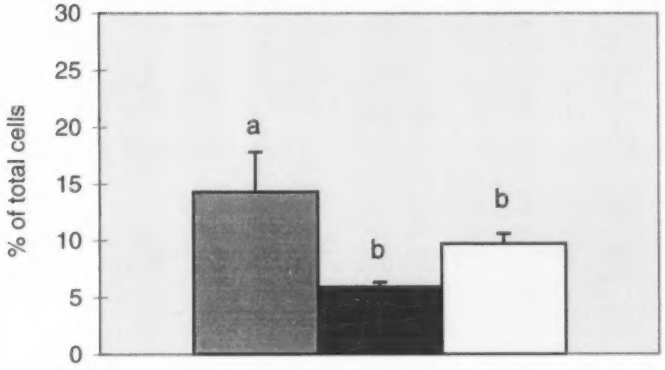


Figure 2.6.8 OX33[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 30 Blood Lymphocyte Phenotypes

Figure 5.4.1 OX19[1,2]

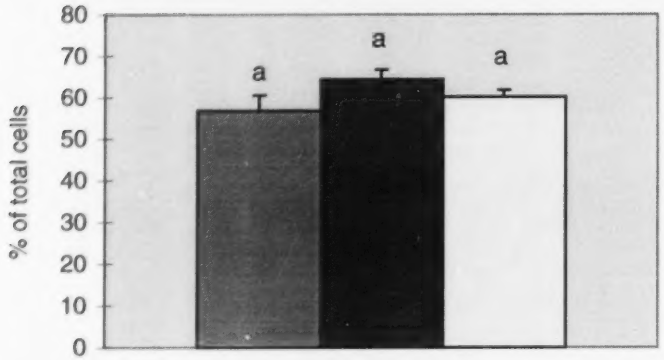


Figure 5.4.2 W3/13[1,2]

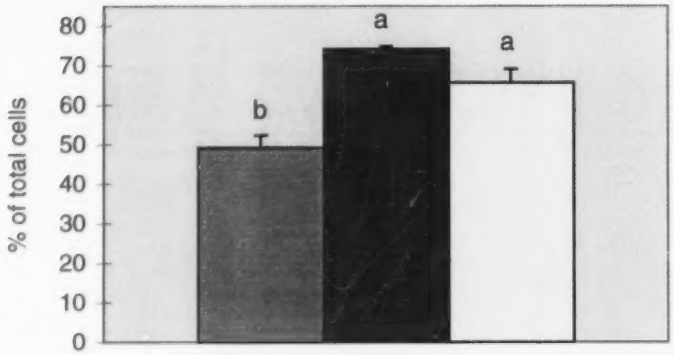
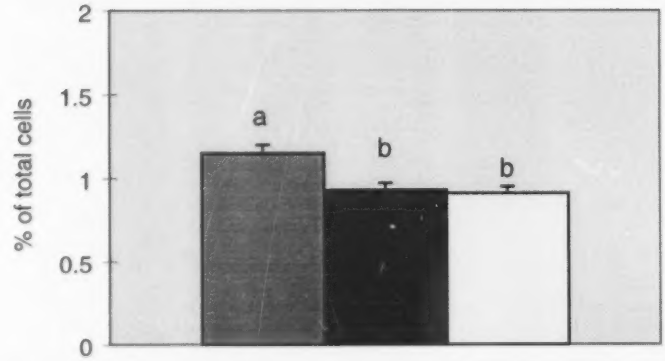


Figure 5.4.3 OX19/W313 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = p>0.05

BBn Day 30 Blood Lymphocyte Phenotypes -(cont)

Figure 5.4.4 W3/25 (CD4)

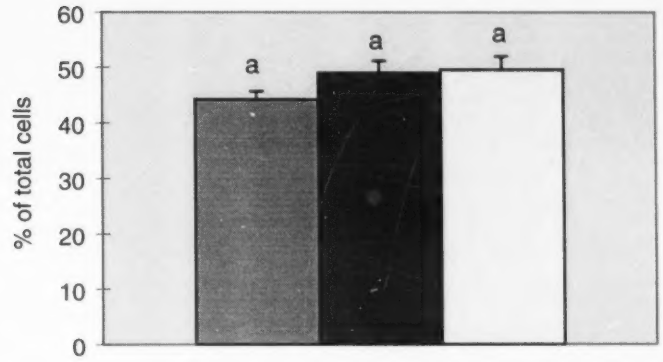


Fig. 5.4.5 OX8 (CD8)

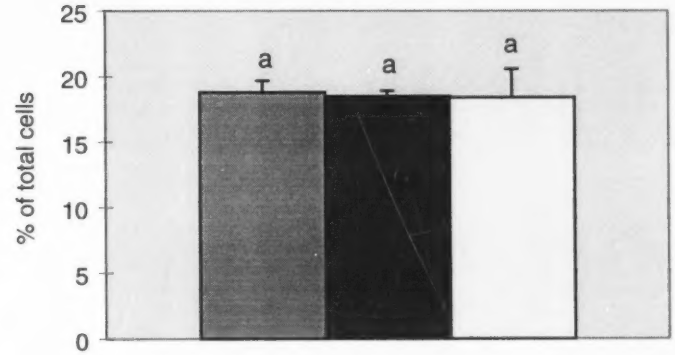
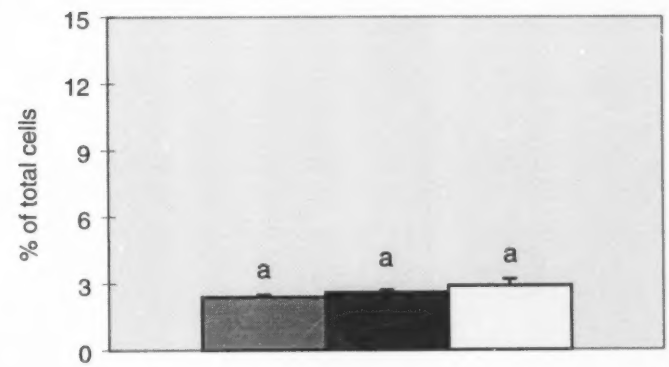


Figure 5.4.6 CD4/CD8 ratio[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 30 Blood Lymphocyte Phenotypes - (cont.)

Figure 5.4.7 OX12[1,2]

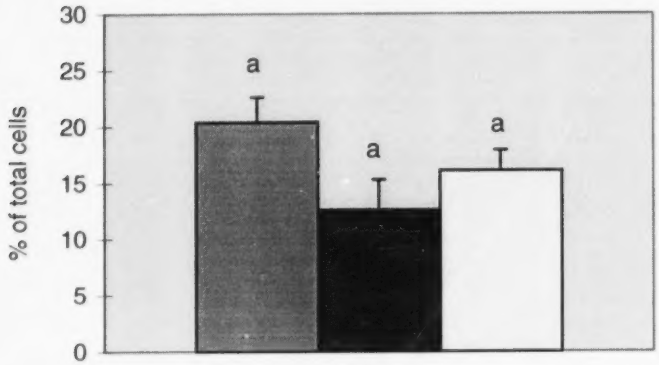


Figure 5.4.8 OX33[1,2]

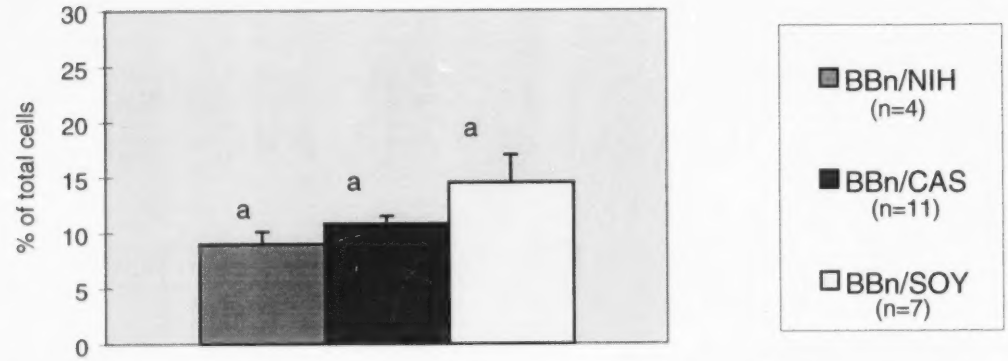
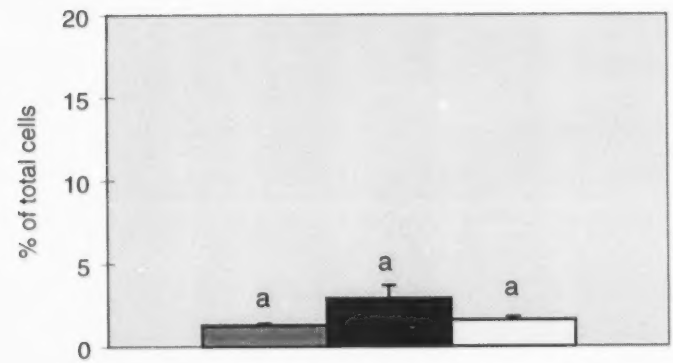


Figure 5.4.9 3·2·3[1,2]

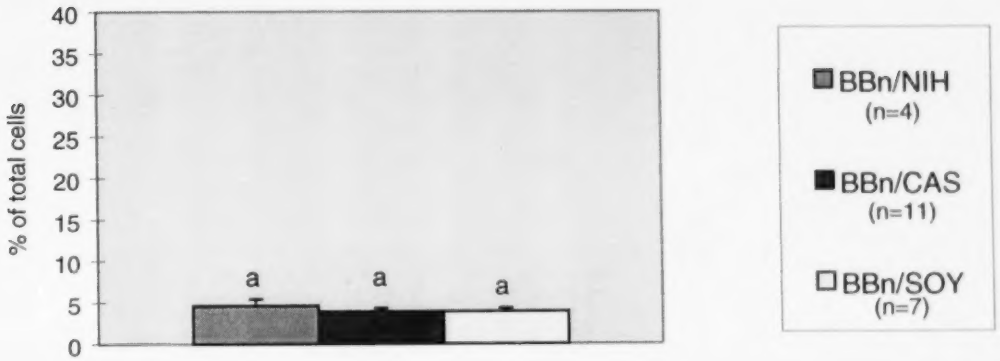


[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

BBn Day 30 Blood Lymphocyte Phenotypes - (cont.)

Figure 5.4.10 OX42[1,2]



[1] Graph represents mean ± SEM for each diet group

[2] Differences were determined by one-way ANOVA, diet groups that do not share a common letter are significantly different. NS = $p>0.05$

Effect of Age and Diet on Splenocyte Phenotypes (BBn)

The effect of age on splenocyte phenotypes was determined for each antibody. The effect of diet on the age differences was only analyzed when at least two diet groups showed changes between ages.

W3/25 (CD4) (Tab. 5.3.1) - All diet groups showed a significant increase (at least $p<0.0006$) in $W3/25^+$ cells between 14 and 30 days of age. The increase in the SOY group was significantly ($p<0.05$) greater than that of the CAS group.

OX8 (CD8) (Tab. 5.3.2) - All diet groups showed a significant increase of $OX8^+$ cells of at least $p<0.006$. There were no differences between diet groups in the magnitude of the changes between days.

CD4/CD8 ratio (Tab. 5.3.3) - Only the NIH group showed a difference in this value between days ($p<0.01$) and there were no significant differences in the magnitude of the changes between diet groups.

OX19 (Tab. 5.3.4) - All three diet groups showed an increase in $OX19^+$ cells between 14 and 30 days of age (at least $p <0.0005$). There were no differences in the magnitude of the increases.

W3/13 (Tab. 5.3.5) - There were significant increases in $W3/13^+$ cells in all diet groups (CAS $p<0.0001$, SOY and NIH $p<0.05$). The CAS group showed a significantly larger increase ($p<0.05$) in the percentage of $W3/13^+$ cells compared to the other two groups.

OX19/W313 ratio (Tab. 5.3.6) - Both the NIH and SOY groups showed a significant increase in this ratio between 14 and 30 days of age (NIH $p<0.002$, SOY $p<0.05$), while the CAS group showed no significant difference between days. The NIH fed groups change over time differed significantly ($p<0.05$) from that of the CAS group, while the SOY group did not differ from the other two.

OX12 (Tab. 5.3.7) - The SOY group was the only group that showed a significant difference between 14 and 30 days of age ($p<0.05$).

OX33 (Tab. 5.3.8) - The CAS diet group was the only group to show a significant difference in the percentage of $OX33^+$ cells between days ($p<0.0001$).

3·2·3 (Tab. 5.3.9) - Only the NIH fed group showed a difference in percentage of $3{\cdot}2{\cdot}3^+$ cells between 14 and 30 days of age ($p<0.0001$).

OX42 (Tab. 5.3.10) - There were no differences in any of the diet groups in the percentage of $OX42^+$ cells between 14 and 30 days of age.

Effect of Age and Diet on Splenocyte Phenotypes (BBn)

Table 5.3.1 W3/25[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	8 ± 1	17 ± 1	p<0.0006	ab
CAS	10 ± 0.7	17 ± 0.7	p<0.0001	b
SOY	7 ± 0.6	23 ± 1	p<0.0001	a

Table 5.3.2 OX8[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	7 ± 1	12 ± 1	p<0.006	a
CAS	9 ± 0.2	14 ± 1	p<0.003	a
SOY	5 ± 0.5	14 ± 0.5	p<0.0001	a

Table 5.3.3 CD4/CD8 ratio[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	1.1 ± 0.06	1.4 ± 0.1	p<0.01	N/A
CAS	1.1 ± 0.05	1.3 ± 0.2	NS	N/A
SOY	1.6 ± 0.1	1.7 ± 0.1	NS	N/A

[1] Values represent mean ± SEM

[2] Significance between days within diets was determined by one-way ANOVA, NS = p>0.05

[3] Significance of diets on age differences was determined when at least two diet groups showed changes over time. This was determined by the SAS GLM procedure and the estimate statement. Diet groups that do not share a common letter are significantly different, NS = p<0.05.

Effect of Age and Diet on Splenocyte Phenotypes (BBn)- (cont)

Table 5.3.4 OX19[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	14 ± 2	40 ± 3	p<0.0001	a
CAS	17 ± 0.4	34 ± 2	p<0.0002	a
SOY	16 ± 2	37 ± 2	p<0.0005	a

Table 5.3.5 W3/13[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	15 ± 2	23 ± 2	p<0.05	b
CAS	17 ± 2	38 ± 1	p<0.0001	a
SOY	18 ± 2	29 ± 1	p<0.05	b

Table 5.3.6 OX19/W313 ratio[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	0.9 ± 0.1	1.8 ± 0.1	p<0.002	a
CAS	1.1 ± 0.2	0.8 ±	NS	b
SOY	0.9 ± 0.1	1.3 ± 0.1	p<0.05	ab

[1] Values represent mean ± SEM

[2] Significance between days within diets was determined by one-way ANOVA, NS = p>0.05

[3] Significance of diets on age differences was determined when at least two diet groups showed changes over time. This was determined by the SAS GLM procedure and the estimate statement. Diet groups that do not share a common letter are significantly different, NS = p<0.05.

Effect of Age and Diet on Splenocyte Phenotypes (BBn)- (cont)

Table 5.3.7 OX12[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	25 ± 2	31 ± 3	NS	N/A
CAS	34 ± 1	28 ± 3	NS	N/A
SOY	24 ± 1	33 ± 1	p<0.05	N/A

Table 5.3.8 OX33[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	22 ± 1	22 ± 2	NS	N/A
CAS	32 ± 2	22 ± 1	p<0.0001	N/A
SOY	25 ± 0.5	28 ± 3	NS	N/A

Table 3.3.9 5·2·3[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	6 ± 1	2 ± 0.5	p<0.0001	N/A
CAS	6 ± 1	5 ± 1	NS	N/A
SOY	3 ± 0.5	4 ± 0.3	NS	N/A

[1] Values represent mean ± SEM

[2] Significance between days within diets was determined by one-way ANOVA, NS = p>0.05

[3] Significance of diets on age differences was determined when at least two diet groups showed changes over time. This was determined by the SAS GLM procedure and the estimate statement. Diet groups that do not share a common letter are significantly different, NS = p<0.05.

Effect of Age and Diet on Splenocyte Phenotypes (BBn)- (cont)

Table 5.3.10 OX42[1]

Diet	Day 14	Day 30	Effect of Age[2]	Effect of Diet on Age Differences[3]
	% of total cells	% of total cells		
NIH	9 ± 3	4 ± 1	NS	N/A
CAS	5 ± 0.4	7 ± 1	NS	N/A
SOY	3 ± 0.5	6 ± 0.5	NS	N/A

[1] Values represent mean ± SEM

[2] Significance between days within diets was determined by one-way ANOVA, NS = $p>0.05$

[3] Significance of diets on age differences was determined when at least two diet groups showed changes over time. This was determined by the SAS GLM procedure and the estimate statement. Diet groups that do not share a common letter are significantly different, NS = $p<0.05$.

Chapter Six - Discussion

Introduction

Previous studies using the BBdp rat have shown that the incidence of the diabetes in these animals can be altered by weaning pups to different, nutritionally complete diets (Elliot & Martin, 1984; Scott et al., 1985; Hoorfar et al., 1992). Pups weaned to non-purified laboratory chow develop diabetes with an incidence rate of 60%-80% (Scott et al., 1994). Pups weaned to a soy or casein based semi-purified (SP) diet have a lower incidence of diabetes, 40%-60% and 20%-30% respectively (Scott et al., 1994). The majority of these studies have focused on incidence rates in animals weaned to different diets and have not examined how feeding different diets may alter the pathogenesis of the disease.

An important factor in determining whether or not diet will affect diabetes incidence in BBdp rats is the timing of introduction of the diet. Protective, semi-purified diets must be introduced prior to 30 days of age and continue for at least 100 days to decrease diabetes incidence (Issa-Cergui et al., 1989; Scott and Marliss, 1991). This evidence points to a "critical period" in the development of the disease. This "critical period" effect has been shown in other types of studies that have examined protection from diabetes through direct immune mechanisms (Greiner et al., 1987; Jaing et al., 1990).

This study, discussed below, is one of the first to compare a wide variety of lymphocyte phenotypes from lymphoid organs and peripheral blood, in rats weaned to different diets. As well, it is one of the only studies to ensure that rat pups were exposed to only one weaning diet, as pups were weaned to diets analogous to their dams. In response to the data suggesting a critical period in the first 30 days of life lymphocytes were examined at two time points within this period. The first was prior to the ingestion of solid food (14 days of age) and the second was at 30 days of age.

As the main objective of this study was to examine the effect of different weaning diets in the BBdp rat, the majority of the discussion focuses on effects seen in this group. Unless otherwise stated, the term rats refers to BBdp rats, in this section. It was hypothesized diet would not effect lymphocyte phenotypes in BBn rats, which served as our controls, however some differences were found. This data

is unique as very little data exists about the effect of early diet in normal animals. BBn animals are discussed specifically at the end of this section.

Animal Characteristics

At 14 days of age some differences were seen between the three diet groups. There were differences in body weight with the NIH group being heavier than the SOY group. Litters were culled to between 8 and 12 pups per litter and this difference may be a residual effect of different litter sizes at birth. Differences were also seen in the BBn animals at this time point, however in these animals the SOY fed rats had a higher average weight than the other two groups. Again, this is likely due to differences in litter size at birth.

The NIH fed group had more splenocytes per gram of spleen as well as more thymocytes per gram of thymus. This may be indicative of a more activated immune system and greater lymphocyte proliferation in these animals, as more cells may be due to lymphocyte proliferation in these animals. In the BBn animals, the number of splenocytes per gram of spleen was also greatest in the animals in the NIH group. Since dam's milk can contain different antigens depending on maternal diet (Hattevig et al., 1990), it is possible that the NIH and SOY diets which contain more foreign (plant) proteins than the CAS diet are providing antigenic stimulus for the immune system in these young animals.

At 30 days of age the same trend was seen in comparisons of body weight. The NIH fed animals weighed significantly more that the other two groups. This is a curious finding since the NIH diet is much less energy dense than the two SP diets. The NIH diet provides the majority of its energy from carbohydrate whereas the CAS and SOY diets are higher in fat. Another difference between SP and chow diets is the amount of complex carbohydrate and fiber. It is speculated that increasing the fiber content of the diet can stimulate the growth of the intestine . Therefore the NIH fed animals may have increased absorptive capacity compared to the other two groups. Since food intake and body composition were not examined it is difficult to determine conclusively the reasons behind the differences in body weights. The potential implications of these differences, in the development of diabetes, will be discussed later.

Spleen

It was expected that no differences would be seen in the spleen phenotypes at 14 days of age since at this time point all nutrients are delivered via the dams milk. However diet did affect total B and T cell populations but not the proportion of $CD4^+$ and $CD8^+$ T cells or the percentages of NK cells or macrophages in spleen.

The BBdp pups from the dams fed the NIH diet had a lower percentage of $OX19^+$ T cells compared to the CAS group but not the SOY group. Analysis with the W3/13 pan T cell marker showed the SOY group had a percentage of positive cells that was lower than the other two groups. This may indicate that diet-induced effects on diabetes incidence and progression, may begin through differences in the cmposition of dam's milk. Therefore it appears that in these rats that are genetically T cell deficient from birth (Yale et al., 1985) alterations to the lymphocyte profile may also occur from birth onward.

Total B cells, determined by positive marking with the anti-bodies OX12 and OX33, showed that the NIH diet group had a lower proportion than the other two groups. It is likely that the three diet groups received different antigens via the dams milk since the dams were ingesting different proteins. It is known that altering maternal diet during lactation will alter the types of dietary antigens found in milk (Hattevig et al., 1990), and that dietary macro-molecules can pass through the gut and be absorbed as intact antigens (Warshow et al., 1971). Different antigens may elucidate different responses in the pups. Soy and cow's milk proteins are considered to be highly antigenic for the production anti-bodies (Bock and Martin, 1983). It may be that these antigens are passing through the gut and stimulating the production of anti-bodies by B cells. This would also lead to the proliferation of B cells. In the BBn animals the CAS diet group also had a higher percentage of both OX12 and OX33 cells. The fact that relative proportions of macrophages and NK cells were not different between diet groups in the BBdp animals supports this theory since these two cell populations are part of the non-specific immune system and they proliferate in response to cellular changes rather than soluble antigen .

Breast milk contains many immune factors (Ogra & Ogra, 1978) and it is possible that feeding the dams different diets changes the quality and/or quantity of the immune components in the milk. Specifically, it has been demonstrated that food specific anti-bodies can be found in human milk (Juto and Holm, 1992) which may

be the result of anti-body secreting cells that have migrated from the mother's gut (Czerkinsky et al., 1987). If this is the case in rats this may influence the development of the immune system in the pups, including their relative lymphocyte populations and their reaction to these antigens later in life.

At 30 days of age, several differences were seen in splenocyte phenotypes. With the two pan-T cell markers, OX19 and W3/13, the NIH fed rats had a significantly lower proportion of $OX19^+$ but not $W3/13^+$ cells compared to the other diets. All three diet groups showed a higher percentage of $OX19^+$ T cells than $W3/13^+$ cells. The OX19 antibody binds to the CD5 antigen on all T cells (Vermeer et al., 1994), while W3/13 binds a thymocyte glycoprotein (Woolet et al., 1985). The difference in the number of $OX19^+$ T cells is likely the most relevant to the future development of diabetes in these animals since BBdp rats, with the lowest $OX19^+$ lymphocyte proportion have been shown to be the most likely to develop diabetes (Marliss et al., 1985).

There is evidence that low T cell counts, characteristic of the BBdp rat are maintained with age due to a reduced expansion of T cells compared to the BBn rat (Yale et al., 1985). The fact that there was no difference between day 14 and day 30 in the proportion of splenocytes that were $OX19^+$ in any of the diet groups supports this evidence. As well the percentage of T cells that were positive for the W3/13 marker dropped significantly between 14 and 30 days of age in all diet groups while this percentage increased in BBn rats. The importance of the lack of expansion in the proportion of T cells is highlighted by the fact that the BBn animals in all diet groups showed an increase in positive cells with both markers, including a 100% increase in $OX19^+$ cells, between 14 and 30 days of age. It should be noted that although none of the BBdp groups showed an increase in OX19 cells with age, at both time points the percentage of T cells positive for this marker was lower in the BBdp animals.

Both the CD4 (helper) and CD8 (suppresser) sub-groups of T cells were significantly lower in the NIH fed group. However, because both groups of cells were reduced there was no difference in the CD4/CD8 ratio between diet groups. The CAS group were not significantly different from the BBn CAS group in the relative proportion of $CD4^+$ cells suggesting a partial correction in the lymphopenia in these animals. As well the CAS fed animals showed a significantly larger increase in the

percentage of W3/25$^+$ T cells between 14 and 30 days of age compared to the other two groups. It does not appear that a complete reversal of the T cell lymphopenia is necessary for prevention of the disease in these animals. Prevention of diabetes by diet (Scott et al., 1985) or silica (Oschilewski et al., 1985) did not the alter the lymphopenia in these animals. It is therefore difficult to know what influence these changes may have on the disease process. It has been demonstrated that both CD4$^+$ and CD8$^+$ T cells are involved in the disease (Edourd et al., 1993, Metroz-Dayer et al., 1990) so it is reasonable to assume that changing the proportions of these cells to those more closely resembling that of the BBn rat may confer protection from the disease.

The relative proportions of B cells that were positive for the B cells markers used, OX12 and OX33, were lowest in the CAS group. The CAS fed rats had a lower proportion of OX12$^+$ cells than the SOY group and a lower proportion of OX33$^+$ cells than the NIH cells. B cells are responsible for the production of antibodies and they proliferate in response to antigen exposure, producing more B cells of the same specificity and anti-body secreting plasma cells (Kuby, 1993). It is possible that the increased proportion of B cells in the NIH and SOY groups represent a population of B cells that is responding to an auto-antigen expressed on the pancreas, since the numbers of these antibodies is increased in animals who become diabetic (Dryberg et al., 1984) and these are the two diet groups most likely to develop the disease. It is also possible that the lower percentage of positive B cells in the CAS group is relative to the higher percentage of T cells in this group. The CAS group showed no difference in the relative proportion of OX33$^+$ B cells between day 14 and day 30 and a smaller increase in the percentage of OX12$^+$ B cells than the other two groups.

The number of natural killer (NK) cells was higher in the CAS group however it does not appear that NK cells are required for the disease process to be initiated. NK cells have been shown to be cytotoxic to β-cells *in vitro* (Nakamura et al., 1990) , however removal of NK cells from adoptive transfer experiments does not affect the incidence rate of the disease (Ellerman et al., 1993). It may be that NK cells are involved in the later stages of the disease however, due to this evidence (Ellerman et al., 1993) it seems unlikely that they are a factor at this age. It has also been shown that NK cells from rats fed a CAS diet are less active than those from animals fed

chow (Field, 1995). Therefore if NK cells are involved in the destruction of β-cells during the latter stages of the disease process, the CAS diet may decrease this activity.

The higher percentage of macrophages in the two SP diet groups may impart some protection to the CAS animals since it has been shown that removal of macrophages from adoptive transfer injections leads to an increase in diabetes incidence in recipients (Metroz-Dayer et al., 1990). This effect has been suggested to be due to nitric oxide produced by macrophages which suppresses the proliferative response of splenocytes in the BBdp rat (Lee, 1994). This suppressive effect of macrophages has also been documented in stressed rats, of other strains (Fleshner M, 1995). Another possibility exists for macrophages to be involved in protection from diabetes development. TNF-α, produced by macrophages may play a protective role against the development of diabetes in these animals at an early age (Satoh et al., 1987). Since the number of macrophages was higher in the two SP fed groups it is possible that TNF-α production is also increased in these animals.

Mesenteric Lymph Nodes

With respect to the T cell markers, differences were seen with both pan-T cell markers (OX19 and W3/13) as well as in the two T cell subgroups, CD4 and CD8. The NIH fed group was lower than the other two groups in the percentage of cells positive for OX19 and was also lower than the CAS group for the percentage of cells positive for the W3/13 marker. All three groups had significantly lower positive percentages for both anti-bodies than their respective diet group in the BBn animals. However this data shows a general trend of the CAS animals having an increased overall T cell percentage over the other BBdp two groups.

The NIH group had a significantly lower relative percentage of $CD4^+$ T cells than the CAS group and a significantly lower relative percentage of $CD8^+$ T cells than the SOY group. The CAS group showed no differences in the percentages of $CD4^+$ and $CD8^+$ cells compared to any of the BBn diet groups, indicating CD4 and CD8 T cell profile that is correlated with protection from diabetes.

Taken together, these differences in lymphocyte populations led to a CD4/CD8 ratio that was higher in the NIH fed animals compared to the other two groups. Since this ratio represents the balance of T cells which initiate and "help" an immune response (CD4 cells) to those which suppress it (CD8 cells), the difference in this ratio may represent a shift towards more suppressive capability in the two SP diet groups. Since it has been postulated than auto-immune disorders including diabetes may be the result of impaired suppression, this ratio difference may impact the development or prevention of diabetes in these animals.

The mesenteric lymph nodes are considered to be part of the gut-associated lymphoid tissue (GALT), and therefore may be directly affected by food antigens. If a cross reaction between a dietary antigen and a pancreatic antigen is in part responsible for the auto-immune process (Karjalainen et al., 1992; Virtanen et al., 1994) this effect may be mediated through GALT since these lymphocytes would be one of the first to be exposed to the antigens. If there is a reduced number of $CD8^+$ cells in the MLN, as described above, this reduced suppression may allow the cross reaction to occur in genetically susceptible rats.

In another study (Fowell & Mason, 1993), it was shown that thoracic duct lymphocytes of rats rendered susceptible to diabetes through lymphocyte depletion were activated and proliferating. Comparison of T lymphocytes from different lymph nodes in these animals showed these T cells likely came from the MLN. It has been suggested that the anitgenic stimulus for these cells came from the gut (Fowell & Mason, 1993).

There were no differences in the proportions of cells which were positive for either of the B cell markers, OX12 or OX33, between any of the diet groups.

Blood

In the BBdp rats in general there was lower binding of all of the T cell antibody markers examined, compared to the BBn animals. Therefore it appears that there is a population of T cells in the peripheral circulation of these animals that cannot be identified by the standard lymphocyte monoclonal antibodies used in this study. This data also demonstrates the importance of studying different populations of lymphocytes in immune related disorders, as it is often presumed that splenocyte

populations are representative of peripheral populations, which is not the case in this instance.

The NIH fed rats had a significantly lower percentage of $OX19^+$ cells than either the CAS or the SOY group and this held true for both the $CD4^+$ and $CD8^+$ cells as well, giving the NIH fed animals a very high CD4/CD8 ratio compared to the other two groups as well as compared to the BBn animals. Both the NIH and SOY groups had a lower percentage of $W3/13^+$ cells than the CAS group.

This data shows that the SP diet groups are still very T cell lymphopenic. However, the ratio of CD4 (helper) to CD8 (suppresser) cells was closer to the BBn animals in these diet groups. Therefore, even though they still have a very low proportion of T cells, their CD4/CD8 ratio may represent a balance that allows a more protective response to occur. The NIH fed group has virtually no circulating $OX8^+$ T cells which supports data from other studies (Yale & Marliss, 1984; Jackson et al., 1983), and it may facilitate the disease process.

One study has shown that virtually all $CD8^+$ cells found in peripheral blood in the BBdp rat are immature and have attenuated life spans (Groen et al., 1996). As well, they may play a role in diabetes development due to a decreased expression of the CD8 receptor compared to normal rats, this low expression may increase their autoreactivity (Groen et al., 1996). It may be that the improvement seen in the CD8 T cell profile of the CAS and SOY animals is due to an increase in normal CD8 cells.

With both B cell markers, OX12 and OX33, the SOY fed animals had significantly more positive cells than either of the other two diet groups. There may be a component of the SOY diet that elicits an antibody response and this may be why these populations are higher in these animals. If it is related to the diabetic process it is difficult to determine why the NIH animals did not have elevated B cell levels as well.

As in the spleen, the CAS animals had significantly more NK cells than either the NIH or SOY groups. Therefore, as was the case in the blood it is possible that the activity of NK cells may be down regulated when animals are fed the CAS diet (Field, 1995), possibly by macrophages, which were increased in the CAS group above the other two.

Summary

Hypothesis one stated that BBdp rats fed diets known to give different diabetes incidence, NIH chow (high incidence), casein based SP diet (low incidence) or soy based SP diet (intermediate incidence), would have different lymphocyte phenotype profiles. Animals fed the chow diet would show the typical BBdp lymphopenic profile. Animals fed the casein based SP diet would show increased numbers of T-cells and those fed the soy based SP diet would be intermediate between the two.

It is clear from the results and discussion above that diet did have an effect on and immune development of the rat pups, as well as on their early growth pattern. The diabetogenic diet (NIH) led to a lymphocyte profile characteristic of BBdp rats that develop diabetes whereas the protective CAS diet led to a lymphocyte profile more closely resembling that of the BBn rat, including an increased number of total T cells and $CD4^+$ and $CD8^+$ T cell sub-groups. The SOY fed animals had a lymphocyte profile intermediate between the other two which corresponds to the intermediate incidence of diabetes in these animals.

Several lines of evidence seem to indicate that a lack of an effective suppresser mechanism against the auto-immune process is responsible for the development of diabetes in these animals. BBn and other rat strains can be rendered susceptible to diabetes by depletion of lymphocyte subsets (Fowell & Mason, 1993; Greiner et al., 1987; Jaing et al., 1990). This observation combined the lymphopenia found in genetically susceptible rats, indicates that potentially auto-reactive cells occur in all rats, but a subgroup of protective cells are usually absent in the BBdp rat. The lymphocyte phenotypes that are most affected by the lymphopenia in the BBdp rat are the $CD8^+$ cells (Yale & Marliss, 1984; Jackson et al., 1983) and T cells expressing the RT6.1 marker (Burstein et al., 1989). It is reasonable to assume that it is one or both of these subgroups that is involved in protection from diabetes.

One study has shown that RT6 expression decreases when T cells are activated and is re-expressed when cells return to a non-activated state. As well RT6.1 is a maturational marker (Burstein et al., 1989) and its absence may indicate

a lack of mature T cells in BBdp rats. CD8+ cells are believed to be suppresser cells (Kuby,1993), and involved in suppression of the immune response.

In our study the total number of T cells as well as the T cell subgroups of $CD4^+$ and $CD8^+$ T cells were increased in virtually all organs at 30 days of age in the BBdp animals fed the CAS based SP diet (protective) compared to the BBdp animals fed the NIH chow diet (diabetogenic). The animals fed the SOY diet did not show consistent results with the phenotype markers. Differences were seen between this group and the other two depending on the phenotype , organ and age examined. This may explain the intermediate incidence of diabetes found in these animals, as only some of the factors involved in disease process may be shifted to the protective type.

In peripheral blood, where the $CD8^+$ lymphopenia in the BBdp rat is most pronounced, the CAS and SOY semi-purified diets significantly increased the percentage of positive cells. It is possible that this increase is the most important change for altering diabetes incidence in these rats.

It is difficult to determine which component(s) of the SP diets may be responsible for altering the lymphopenia in these animals. It is possible that it is not a specific component that is responsible but rather a systemic effect of the diets. In our study and in others (Elliot & Martin, 1984; Hoorfar et al., 1992), it was shown that animals fed chow grow more rapidly early in life than those fed the SP diets. This difference disappears by six weeks of age (42 days). It has also been demonstrated that in a given colony of BBdp rats fed chow, those with the highest body weights between 10 and 40 days are at an increased risk for development of diabetes (Pederson et al., 1994). Therefore it seems reasonable to assume that part of the diabetogenic effect of chow may be due to its effect of increasing growth during this critical period in the development of diabetes.

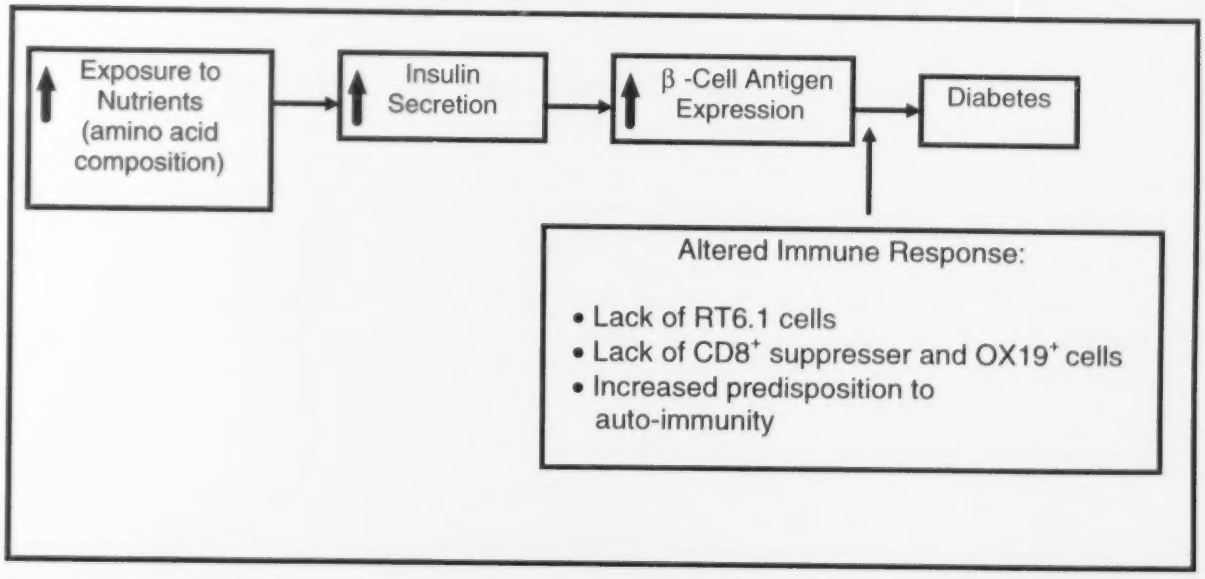
It is interesting that the NIH diet leads to higher rates of growth in these animals. The NIH diet is less energy dense than the CAS or SOY diets therefore the rats fed the NIH diets must have eaten more food or absorbed nutrients with greater efficiency. Studies have shown that increased insulin secretion by cyclophosphamide treatment accelerates β-cell destruction (Mordes et al., 1987). As well, administration of exogenous insulin (which decreases pancreatic secretion of

insulin) can delay the onset of the disease (Gottlieb et al., 1991), and reduction of pancreatic insulin secretion by diazoxide prevents diabetes in the BBdp rat (Vlahos & Yale, 1987). It is possible these results are attributalbe to increased β-cell antigen expression associated with icreased insulin secretion (Appel et al., 1989). Since the autoimmune process in diabetes is directed against the β-cells, this increased antigen expression may increased the likelihood of an auto-immune attack. It is also possible that the different diets may affect insulin secretion due to differences in their amino acid compositions or in their effect on the hormonal response in the animal.

Since the BBn animals also showed differences in body weights at 30 days of age it appears that this effect on early growth is not restricted to BBdp animals. It is also evidence that it is not increased early weight gain alone which leads to the development of diabetes in these animals.

It may be that in BBdp rats fed chow, increased β-cell antigen expression due to increased exposure to nutrients combined with the altered immune system of the BBdp rat to produce auto-immune β-cell destruction in these animals. Protection in animals fed a SP diet may be conferred by two factors, decreased growth and insulin secretion as well as a more protective immune response (See figure 6.1).

Figure 6.1


Exposure to Nutrients (amino acid composition)
Insulin Secretion
β -Cell Antigen Expression
Diabetes
Altered Immune Response:
• Lack of RT6.1 cells
• Lack of CD8+ suppresser and OX19+ cells
• Increased predisposition to auto-immunity

Hypothesis two stated that no differences would be seen in BBn animals fed the different diets. This hypothesis was not supported by our data as differences were seen in lymphocytes from animals fed the different diets. This data is unique as studies that have examined phenotypes in BBn animals have studied them at older ages (Field, 1995) and have not found differences. This study has determined that diet can affect lymphocyte phenotypes in BBn animals at a young age.

The most interesting finding at day 14 was that the relative percentage of both $OX12^+$ and $OX33^+$ cells were increased in the CAS fed animals over the other two diet groups. It is difficult to determine why this would occur. It is known that dietary antigens can be expressed in milk (Hattevig et al., 1990). As stated above, cow' s milk is considered to be highly antigenic for the production of antibodies (Bock and Martin, 1983). The higher proportion of B cells in these animals may therefore be representative of a population of stimulated B cells producing anti-bodies against cow's milk proteins.

At 30 days of age there were no differences in B cell numbers but all three groups differed from each other in their proportions of W3/13 cells, with the CAS fed animals having the highest percentage of positive cells and the NIH fed animals the lowest..

In mesenteric lymph nodes there was only one difference between diet groups. The two SP groups had lower relative percentages of OX33 B cells compared to the NIH group. CD45RA, which is the antigen that OX33 binds to, is not expressed on memory B cells (Hamann, 1992). Since there was no difference between groups in their OX12 populations the differences may represent a population of memory B cells. This could in part be due to the higher number of B cells seen in the spleen of the SP fed rats at 14 days of age, which may have represented a stimulated B cell population.

Only one difference was seen in the blood lymphocyte phenotypes of BBn rats at 30 days of age. The NIH group had a lower percentage of W3/13+ cells than the other two groups. This corresponds to the spleen populations of these animals where the NIH group had a lower $W3/13^+$ count as well.

It is difficult to determine why differences were seen in the BBn animals. Very little research has been done examining immune parameters of animals (or humans) fed different diets as infants. Since casein and soy are very common as protein

sources for infant formulas it is important to realize that feeding different protein sources to infants may affect their immune system early in life, and this could have implications as they age.

Future Research

It is known that weaning diet can influence the development of diabetes in BBdp rats and that weaning to a chow diet can increase diabetes incidence in BBdp rats. The discussion and summary described possible processes by which diet may exert its effect, based on results from our study as well as others.

Future studies examining the relationship between weaning diet and diabetes incidence in the BBdp rat could incorporate some of the ideas discussed above. One possibility would be to look at β-cell expression in rats fed different diets, to determine if this is increased in rats weaned to chow diets compared to those on SP diets.

Studies of different immune parameters could also be examined in these rats. Phenotyping with monoclonal antibodies marking for activation would give useful information about the functional state of lymphocytes from rats fed different diets. Functional studies, such as proliferative responses to mitogen stimulation could also be examined. Cytokine levels in these rats may also elucidate possible mechanisms by which diet mediated differences occur. TNF-α and IL-1, which have been thought to be involved in the disease process could be examined in these animals.

Some of these experiments have been done previously however there is little data on BBdp rats prior to 30 days of age, and less data on this group of animals related to diet changes.

Ultimately, research in this area should guide us to understanding more about insulin dependent diabetes mellitus in humans. The research presented in this thesis has potential to be applied to the human disease. As stated in the introduction, there appears to be an increased risk of disease in formula fed infants compared to those who are breast fed (Mayer et al., 1988; Kostraba et al., 1992). This may be due to similar effects of diet seen in the BBdp rat in this study.

Chapter Seven - Bibliography

Abbas A, Lichtman AH & Pober Js. (1991). Chapter Nineteen - Self Tolerance and Autoimmunity. In:Cellular and Molecular Immunology p. 383, W.B Saunders Company. Philidelphia.

Abbas A, Lichtman AH & Pober Js. (1991). Chapter Twenty -Immune-Mediated Tissue Injury and Disease. In:Cellular and Molecular Immunology pp. 404-407, W.B Saunders Company. Philidelphia.

Alviggi L, Johnston C, Hoskins PJ, Tee DEH, Pyke DA and Leslie RDG et al.. Pathogenesis of insulin-dependent diabetes; a role for activated T-lymphocytes. Lancet, 1984;2:4-6.

Appel MC, Dotta F, O'Neil JJ, Eisenbarth GS. B-cell activity regulates the expresion of islet antigenic determinants. Diabetologia, 1989;32:461 (Abstract).

Barnett AH, Eff C, Leslie RDG and Pyke DA. Diabetes in identical twins. Diabetiologia, 1981;20:87-93.

Baekkeskov S, Aannstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, de Camilli P. Identification of the 64 K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature, 1990;347:151-156.

Blom L, Dahlquist G, Nystrom L, Sandstrom A and Wall S. The Swdish childhod diabetes study - social and perinatal determinants for diabetes in childhood. Diabetologia, 1989;32:7-13.

Bock SA and Martin M. The incidence of adverse reactions to food in infants and children. J Allergy Clin Immunol, 1983;74:98-103.

Borch-Johnsen K, Mandrup-Poulsen T, Zachau-Christiansen B, Joner G, Christy M and Kastruo K. Relation between breast-feeding and incidence rates of insulin-dependent diabetes mellitus - a hypothesis. Lancet, 1984;2: 1083-1086.

Borg LAH, Gagliero C, Sandler S, Welsh N and Eizirik DL. Interleukin-1β increases the activity of superoxide dismutase in rat pancreatic islets. Endocrinology, 1992;130:2851;2857.

Brogren CH, Hoorfar J, Lassen A and Buschard K. Polyclonal activation by dietary immunogens and mitogens: a possible nutritional regulator for onset of diabetes in BB rats. Diabetologia, 1988;37:474A (abstract 70).

Brogren CH, Hoorfar J, and Buschard K. Early weaning and immunogenic dietary proteins as important initiating factors for onset of diabetes in BB-rats. Diabetologia, 1989;32:470A (abstract 64).

Burstein D, Mordes JP, Greiner DI, Stein D, Nakamura N, Handler ES et al. Prevention of diabetes in the BB/Wor rat by a single transfusion of spleen cells:Parameters that affect the degree of protection. Diabetes, 1989;38:24-30.

Casali P, Nakamura M, Ginsberg-Fellner F and Notkins AL. Frequency of B cells committed to the production of antibodies to insulin in newly diabnosed patients with insulin-dependent diabetes mellitus and generation of high affinity human monoclonal IgG to insulin. J Immunol, 1990;144(10):3741-3747.

Castano L and Eisenbarth GS. Type-I diabetes: a chronic autoimmmune disease of human, mouse and rat. Ann Rev Immunol, 1990;8:647.

Cavallo MG, Baroni MG, Toto A, Gearing AJH,Forsey T, Andreani D, Thorpe R and Pozzilli P. Viral infection induces cytokine release by beta islet cells. Immunology, 1992;75:664-668.

Cheung R, Karjalainen J, Vandermeulen J, singal DP and H-M Dosch. T cells from children with IDDM are sensitized to bovine serum albumin. Scand J Immunol, 1994;40:623-628.

Colle E, Fuks A, Poussier P, Edourd P and Guttman RD. Polygenic nature of spontaneous diabetes in the rat - Permissive MHC haplotype and presence of the lymphopenic trait to the BB rat are not sufficient to produce susceptibility. Diabetes, 1992;41:1617-1623.

Colle E, Guttman RD and Seemayer TA. Spontaneous diabetes mellitus syndrome in the rat. 1. Association with the major histocompatibility complex. J Exp Med, 1981;134:1237-1242.

*Czerkinsky C, Prince SJ and Michalek SM. IgA antibody-producing cells in the peripheral blood after antigen ingestion: evidence for a common mucosal immune system in humans. Proc Natl Acad Sci USA, 1987;84:2449-2453.

Dahl-Jorgensen K, Joner G and Hanssen KF. Relationship between cows' milk consumption and incidence of IDDM in childhood. Diabetes Care, 1991; 14(11):1081-1082.

Dahlquest G, Savilahti E and Landin-Olsson M. An increased level of antibodies to β-lactoglobulin is a risk determinant for early-onset Type1 (insulin-dependent) diabetes mellitus independent of islet cell antibodies and early introduction of cow's milk. Diabetologia, 1992;35:980-984.

Daneman D, Fishman L, Clarson C and Martin JM. Dietary triggers of insulin-dependent diabetes in the BB rat. Diabetes Res, 1987;5:93-97.

Dean BM, Bone AJ, Varey AM and Walker R. Insulin autoantibodies, islet cell surface antibodies and the development of spontaneous diabetes in the BB/Edinburgh rat. Clin Exp Immunol, 1987;69:308-313.

Dewey KG, Heinig MJ, Nommsen LA, Peerson JM, Lonnerdal B. Growth of breast-fed and formula-fed infants from 0 to 18 months: the DARLING Study. Pediatrics, 1992;89(6 pt 1):1035-1041.

Dorman JS, LaPorte RE, Trucco M. Genes and environment. Bailliere's Clinical Endocrinology and Metabolism, 1991;5(2):239-245.

Dyrberg T, Poussier P, Nakhooda AF, Marliss EB and Lernmark A. Islet cell surface and lymphocyte antibodies often precede the in the spontaneous diabeties in the BB rat. Diabetologia 1994;26:159-165.

Elliot RB and Martin JM. Dietary protein: A trigger of insulin-dependent diabetes in the BB rat?. Diabetologia, 1984;6:297-299.

Elzirik DL, Sandler S, Welsh N, Cetkovic-Cvrtje M, Nieman A, Geller DA, Pipeleers DG, Bendtzen K and Hellerström C. Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. J Clin Invest, 1994;93:1968-1974.

Elzirik DL, Korbutt GS and Hellerström C. Prolonged exposure of human pancreatic islets to high glucose concentrations in vitro impairs the β-cell function. J Clin Invest, 1992;90:1263-1268.

Ewel CH, Sobel DO, Zeligs Bjand Bellanti JA. Poly I:C accelerates development of diabetes mellitus in diabetes-prone BB rat. Diabetes, 1992;41:1016-1021.

Fava D, David R, Leslie G and Pozzilli P. Relationship between dairy product consumption and incidence of IDDM in childhood in Italy. Diabetes Care, 1994; 17(12):1488-1490.

Fleshner M, Bellgrau D, Watkins LR, Laudenslager ML, Maier SF. Stress-induced reduction in the rat mixed lymphocyte reactionis due to macrophages and not to changes in T cell phenotypes. J Neuroimmunol, 1995;56(1):45-52.

Fowell D and Mason D. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the $CD4^+$ T cell subset that inhibits this autoimmune potential. J Exp Med, 1993;177:627-636.

Fuks A, Colle E, Ono S, Prud'homme G, Seemayer T and Guttmant RD. Immunogenetic studies of insulin-dependent diabetes in the BB rat. Frontiers in Diabetes Research. Lessons from Animal Diabetes.

Gamble DR. The epidemiology of insulin dependent diabetes mellitus with particular reference to the relationship of virus infection to etiology. Epidemiol Rev, 1980;2:49-70.

Gadd S, Silink M, Kamath KR and Skerritt JH. Co-existence of coeliac disease and insulin-dependent diabetes mellitus in children: screening sera using an ELISA test for gliadin antibody. Aust NZ J Med, 1992;22:256-260.

Gerstein HC. Cow's milk exposure and Type I diabetes mellitus - A critical overview of the clinical literature. Diabetes Care, 1994;17(1):13-19.

Gorsuch AN, Spencer KM, Lister J, McNally JM, Dean BM, Bottazzo GF, Cudworth AG. Evidence for a long prediabetic period in Type 1 (insulin-dependent) diabetes mellitus. Lancet II, 1981;1362-1365.

Gottlieb PA, Handler ES, Appel MC, Greiner DL, Mordes JP and Rossini AA. Insulin treatment prevents diabetes mellitus but not thyroiditis in RT6-depleted diabetes resistant BB/Wor rats. Diabetologia, 1991;344:296-300.

Greiner DL, Mordes JP, Handler ES, Angelillo M, Nakamura N and Rossini AA. Depletion of $RT6.1^{+}$ lymphocytes induces diabetes in resistant Biobreeding/Worcester (BB/W) rats. J Exp Med, 1987;166:461-475.

Hattevig G, Kjellman B, Sigurs N, Grodzinsky E, Hed J and Bjorksten B. The effect of maternal avoidance of eggs, cow's milk, and fish during lactation on the development of IgE, IgG, and IgA antibodies in infants. J Allergy and Clin Immunol, 1990;85(1):108-115.

Heinig MJ Nommsen LA, Peerson JM, Lonnerdal B and Dewey KG. Energy and protein intakes of breast-fed and formula-fed infants during the first year of life and their association with growth velocity: The DARLING Study. Am J Clin Nutr, 1993;58(2):152-161.

Helquist S, Plaa BS, Johannesen J and Nerup J. Heat shock protein induction in at pancreatic islets by recombinant human interleukin 1 β. Diabetologia, 1991;34:150-156.

Hoorfar J, Buschard K and Brogren CH. Impact of dietary protein and fat source on the development of insulin-dependent diabetes in the BB rat. Diabetes Research, 1992;20:33-41.

Hoorfer J, Scott FW and Coutier HE. Dietary plant materials and develpment of diabetes in the BB rat. J Nutr, 1991;121:908-916.

Haung X, Hultgren B, Dybdal N and Stewart TA. Islet expression of interferon-α preceds diabetes in both the BB rat and streptozotocin-treated mice. Immunity, 1994;1:469-478.

Issa-Cergui B, Buttmann RD, Seemayer TA, Kelley BE and Colle E. The effect of diet on the spontaneous insulin dependent syndrome in the rat. Diab Res, 1989;9:81-86.

Jackson R, Kadison P, Buse J, Rassi N, Jegasothy B, Eisenbarth GS. Lymphocyte abnormalities in the BB rat. Metab Clin Exp, 1983; 32 (Suppl 1): 83-86.

Jakobsson I. Food antigens in human milk. Eur J Clin Nutr, 1991;45(Suppl. 1):29-33.

Jiang Z, Handler ES, Rossini AA and Woda BA. Immunopathology of diabetes in the RT6-depleted diabetes-resistant BB/Wor rat. Am J Pathol, 1990;137:767-777.

Juto P and Holm S. Gliadin-specific and cow's milk protein-specific IgA in Human Milk. J Ped Gastro Nutr, 1992;15:159-162.

Karjalainen J, Martin JM, Knip M, Ilonen L, Robinson BH, Savilahti E, Akerblom H, Dosch H-M. A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. N Engl J Med,1992; 327: 302-307.

Kawazu S, Suzuki M, Negishi K, Yanagisawa M, Hara Y, Katayama S, Ishii J and Komeda K. Cellular immunity and NK activity in the BB/Wor rat. (1988) In:Frontiers in diabetes research. Lessons from animal diabetes II. (Shafrir F and Renold AE, eds),pp. 68-73, John Libbey &Company, USA.

Kostraba JN, Dorman JS, LaPorte RE, Scott FW, Steenkiste AR, Gloninger M, Drash AL. Early infant diet and risk of IDDM in blacks and whites - a matched case-control study. Diabetes Care, 1992;15(5):626-631.

Kostraba JN, Cruickshanks KJ, Lawler-Heavner J, Jobim LF, Rewers MJ, Gay EC, Chase HP, Klingensmith G and Hamman RF. Early exposure to cow's milk and solid foods in infancy, genetic predisposition and risk of IDDM. Diabetes, 1993;42:288-295.

LaPorte RE, Tajima N, Akerblom HK . Geographic differences in the risk of insulin-dependent diabetes mellitus: the importane of registries. Diabetes Care, 1985; 8 (suppl 1):101-107.

Lee K. Nitric oxide produced by macrophages mediates suppression of Con-A-induced proliferative responses of splenic leukocytes in the diabetes-prone BB -rat. Diabetes, 1994;43:1218-1220.

Like AA. Depletion of RT6.1^{+} T lymphocytes alone is insufficient to induce diabetes in diabetes-resistant BB/Wor rats. Am J Pathol, 1990;136:565-574.

Like AA, Kislauskis E, Williams RM and Rossini AA. Neonatal thymectomy prevents spontaneous diabetes mellitus in the BB/W rat. Science, 1982;216:644.

Like AA, McGill PD and Sroczynski E. Adult thymectomy prevents diabetes mellitus in BB/W rats. Diabetologia, 1986;29:565a (Abstr.)

Lorini R, De Amici M, d'Annunzio G, Vitali L and Scaramuzza A. Low serum levels of tumor necrosis factor-alpha in insulin -dependent diabetic children. Horm Res, 1995;43:206-209.

Lowrie DB. What goes wrong with the macrophage in silicosis? Eur J Respir Dis, 1982;63:180-182.

MacKay P, Jacobson J and Rabinovitch A. Spontaneous diabetes mellitus in the Bio-Breeding Rat - evidence in vitro for natural killer cell lysis of islet cells. J Clin Invest, 1986;77:916-924.

Marliss EB, Grose M and Yale JF. OX19-negative lymphocytes and appearance of diabetes in the BB rat. Abstract. Diabetes, 1985;34(Suppl.1);66A.

Mayer EJ, Hamman RF, Gay EC, Lezotte DC, Savitz DA and Klingensmith GJ. Reduced risk of IDDM among breast-fed children - the Colorado IDDM registry. Diabetes,1988;37:1625-1632.

Metroz-Dayer M, Mouland A, Brideau C, Duhamel D and Poussier P. Adoptive transfer of diabetes in BB rats induced by CD4 T lymphocytes. Diabetes, 1990;39:928-932.

Mimura G. Present status and future view of the genetic study of diabets mellitus. In Mimura G, Baba S, Goto Y, Kobberling J, eds. Clinico-genetic genesis of diabetes mellitus. Amsterdam: Excerpta Medica, 1982:xiii-xxviii. (International congress series 597).

Meuller C, Imboden MA, Hess MW, Laissue JA and Carnaud CC. TNF-α and insulin-dependent diabetes mellitus. Clin Exp Immunol, 1992; 87:237-245.

Muser (Menser)MA, Forrest JM, Bransby RD. Rubella infection and diabetes mellitus. Lancet, 1978;1:57-60.

Mordes JP, Desemone J and Rossini AA. The BB rat. (Review). Diab-MetRev, 1987;3(3):725-750.

Nakamura N, Woda BA, Tafuri A, Greiner DL, Reynolds CW, Ortaldo J, Chick W, Handler ES, Mordes JP and Rossini AA. Intrinsic cytotoxicity of natural killer cells to pancreatic islets in vitro. Diabetes, 1990;39:836-843.

Nerup J and Lernmark A. Autoimmunity in insulin-dependent diabetes mellitus. Am J Med, 1981;70:135-141.

Ogra SS and Ogra PL. Immunologic aspects of human colostrum and milk. I. Distribution characteristics and concentrations of immunoglobulins at different times after the onset of lactation. J Pediatrics, 1978;92(4):546-549.

Ohno Y, Aoki N and Nishimura A: In vitro production of interleukin-1, interleukin-6 and tumor necrosis factor-α in insulin-dependent diabetes mellitus. J Clin Endocrinol Metab, 1993;77:1072-1077.

Oschilewski U, Keisel U and Kolb. Administration of silica prevents diabetes in BB-rats. Diabetes, 1985;34:197-199.
Owerbach D, Lernmark AA, Platz P et al. HLA-D region beta-chain DNA endonuclease fragments differ betwen HLA-DR identical healthy and insulin-dependent diabetic individuals. Nature, 1983;303:815-817.

Parfrey NA, Prud'homme GJ, Colle E, Fuks A, Seemayer TA and Guttman RD. Immunologic and genetic studies of diabetes in the BB rat. Critical Rev Immunol, 1989;9(1):45-65.

Pathak A, Shah N and Tataria A. Growth of exclusively breastfed infants. Indian Pediatr, 1993;30(11):1291-1300.

Peakman M, Leslie RD, Vergani D. Immunological studies on type 1 diabetes in identical twins. [Review]. Arch Dis Child, 1993;69(1):97-99.

Pedersen CR, Bock TS, Hansen SV, Hansen MW and Buschard K. High juvenile body weight and low insulin levels as markers preceding early diabetes in the BB rat. Autoimmunity, 1994;17:261-269.

Reunanen A, Akerblom H. A national drug register as a data source in the study of the epidemiology of IDDM in children in Finland. In:Serrano-Rios M, Lefebvre PJ, eds. Diabetes. Amsterdam: Elsevier Science, 1985.

Robinson BH, Dosch H-M, Martin JM, Akerblom H, Savilahti E, Knip M and Ilonen J. A model for the involvement of MHC class II proteins in the development of Type 1 (insulin-dependent) diabetes mellitus in response to bovine serum albumin peptides. 1993; 36:364-368.

Roche AF, Guo S, Siervogel RM, Khamis HJ and Chandra RK. Growth comparison of breast-fed and formula-fed infants. Can J Public Health, 1993; 84(2):132-135.

Rossini AA, Mordes JP, Greiner DL, Nakano K, Appel MC, Handler ES. Spleen cell transfusion in the BB/W rat: Prevention of diabetes, MHC restriction, and long term persistence of transfused cells. J Clin Invest, 1986;77:1399-1401.

Rothe H, Fehsel K and Kolb H. Tumour necrosis factor alpha production is upregulated in diabetes prone BB rats. Diabetologia, 1990;33:573-575.

Sandler S, Eizirik DL, Svensson C, Strandell E and Welsh M. Biochemical and molecular actions of interleukin-a and insulin secretion. Diabetes, 1991;10:241-253.

Satoh J, Seino H, Shintani S, Tanaka S, Ohteki T and Masuda T. Inhibition of type 1 diabetes in BB rats with recombinant human tumor necrosis factor- alpha. J Immunol, 1990;145:1395-1399.

Saurez-Pinchon WL, Strynadka K, Schulz R and Rabinovitch A. Mechanisms of cytokine-induced destruction of rat insulinoma cells: The role of nitric oxide. Endocrinology, 1994;134:1006-1010.

Scott FW, Cui J and Rowsell P. Food and the development of autoimmune disease. Trends in Food Sci Tech, 1994;

Scott FW and Marliss EB. Conference summary: Diet as an environmental factor in the development of insulin-dependent diabetes mellitus. Can J Physiol Pharmacol, 1991;69:311-319.

Scott FW, Mongeau R, Kardish M, Hatina G, Trick KD and Wojcinski Z. Diet can prevent diabetes in the BB rat. Diabetes, 1985;34:1059:1062.

Svejgaard A, Platz P and Ryder LP. HLA and disease 1982 - a survey. Immunol Rev, 1983;70:193-218.

Ternick T and Avrameas S. Murine natural monoclonal autoantibodies: a study of their polyspecificity and their affinities. Immonol Rev, 1986;94:99.

Thomson G, Robinson WP, Kuhner MK et al. Genetic heterogeneity, models of inheritance, and risk estimates for a joint study of Caucasions with insulin-dependent diabetes mellitus. Am J Human Gen, 1988;43:799-816.

Thorsby E, Gjertsen HA, Lundin KEA, Ronnigen KS. Insulin dependent diabetes mellitus susceptibility or protection may be determined by certain HLA-DQ molecules. Bailliere's Clinical Endocrinology and Metabolism, 1991;5(2):361-373.

Todd JA, Bell JI and McDevitt HO. HLA-DQβ gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus Nature, 1987;329:599-604..

Tiwari JL and Terasaki PI. Endocrinology-juvenile diabetes mellitus (insulin-dependent) i

Tun RYM, Peakman M, Alviggi L, Hussain MJ, Lo SSS, Shattock M, Pyke DA, Bottazzo GF, Vergani D and Leslie RDG. Importance of persistent cellular and humoral immune changes before diabetes develops: prospective study of identical twins. BMJ, 1994;308:1063-1068.

Vermeer LA, de Boer NK, Bucci C, Bos NA, Kroese FG and Alberti S. MRC OX19 recognizes the rat CD5 surface glycoprotein, but does not provide evidence for a population of CD5 bright B cells. Eur J Immunol, 1994;24(3):585-592.

Virtanen SM, Saukkonen T, Savilahti E, Ylonen K, Rasanen L, Aro A, Knip M, Tuomilehto J, Akerblom HK and the Childhood Diabetes in Finland Study Group. Diet, cow's milk protein antibodies and the risk of IDDM in Finnish children. Diabetologia, 1994;37:381-387.

Vlahos WD and Yale JF. Prevention of Type I diabetes in the BB rat by diazoxide. Clin Invest Med, 1987;10;B65.

Warshaw AL, Walker WA, Cornell R and Isselbacher KJ. Small intestinal permeability to macromolecules. Transmission of horseradish peroxidase into mesenteric lymph and portal blood. Lab Invest, 1971;25:675-683.

Wertman E, Zilber N and Abramsky O. An association between multiple sclerosis and type I diabetes mellitus. J Neurol, 1992;(1):43-45

Whalen BJ, Greiner DL, Mordes JP and Rossini AA. Adoptive transfer of autoimmune diabetes mellitus to athymic rats: Synergy of CD4+ and CD8+ T cells and prevention by RT6+ cells. J Autoimmunol, 1994;7:819-831.

Wilkin T and Armitage M. Markers for insulin-dependent diabetes: Towards early dectection. Br Med J, 1986;108(1) :1-12.

Wogensen LD, Kolb-Bachofen B, christiansen P, Dinarello CA, Mandrup-Poulsen T , Martin S and Nerup J. Functional and morphologica effects of interleukin-1β on the perfused rat pancreas. Diabetologia, 1990;(33):15-23.

Woollett GR, Barclay AN, Puklavec M and Williams AF. Molecular and antigenic heterogeneity of the rat leukocyte-common antigen from thymocytes and T and B lymphocytes. Eur J Immunol, 1985;15:168-173.

Yale JF and Marliss EB. Altered immunity and diabetes iin the BB rat. Clin Exp Immunol, 1984;57:1-11.

Yoon JW, Autin M, Onodera T and Notkins AL. Virus induced diabetes mellitus:isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med, 1979;300:1173-1179.